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1. INTRODUCTION

The innate immune system (IS) is the first to be activated by invading pathogens and eventually sufficient to clear the infection.[1] However, the adaptive IS is triggered when the innate IS is overwhelmed. Hence, specific B- and T-cell activation happens for pathogen clearance.

In recent years remarkable progress was made in our understanding how the innate IS recognizes microbial invaders and how this identification leads to an appropriate activation of signaling pathways that culminate in the transcriptional regulation of immune response genes including pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, type 1 interferon, antimicrobial peptides, and adhesion molecules.[2,3]

It was discovered that several classes of germline-encoded pattern recognition receptors (PRRs) play an important role in innate immunity. PRRs recognize pathogen-associated molecular patterns (PAMPs) including lipids, microbial DNA, polysaccharides, and proteins forming bacterial flagella, which cannot be found in the host organism. Moreover, endogenous stress signals termed danger associated molecular patterns (DAMPs) like uric acid crystals or DNA can also be sensed by PRRs. Four different types of PRRs are known: the membrane bound toll-like receptors (TLRs) and C-type lectins (CLT) and the intracellular RIG-like receptors (RLRs) like RIG-1 and MDA5, and NOD-like receptors (NLRs). [4]

1.1. NOD-like receptors

NLRs are expressed mainly in immune cells like lymphocytes and antigen-presenting cells (APCs) such as dendritic cells and macrophages. This fact evidences the importance of NLRs in immune defense; however, NLRs can also be found in non-immune cells, including epithelial and mesoepithelial cells which form the first barrier of defense in human tissue. Stimulation by PAMPs or DAMPs of intracellular NLRs leads to an activation of downstream signaling pathways for the production of pro-inflammatory cytokines such as IL-β and IL-18.[3,5]

Structurally, NLRs are multidomain proteins with a tripartite architecture, which are characterized by the presence of a central nucleotide domain (NACHT) typically flanked by C-terminal leucine rich repeats (LRRs) and N-terminal caspase recruitment (CARD) or pyrin (PYD) domains.[3,4]

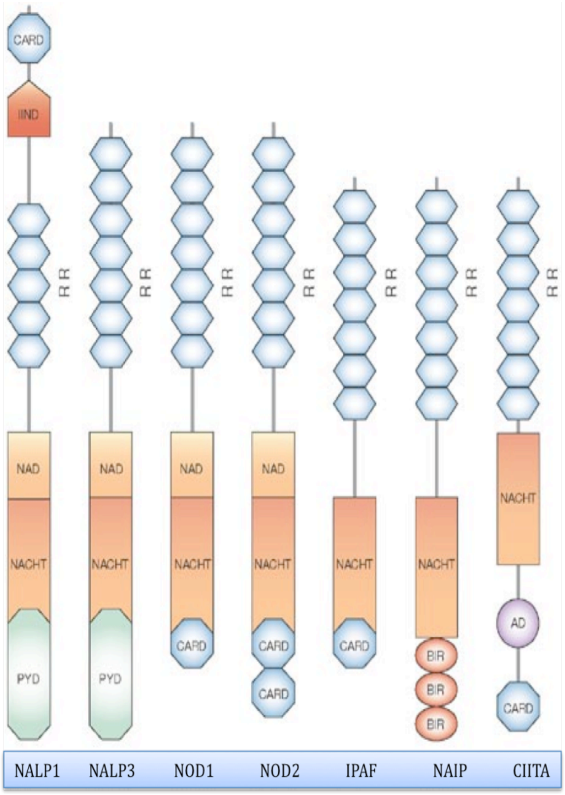

Figure 1 | Structure of NLR-family members. Structural differences of NLR-family members. The only shared domains are the NACHT domain and the LRRs. Picture taken from Church *et al*.[6] Abbreviations: AD-activation domain; BIR-baculovirus; CARD-caspase-recruitment domain; CIITA-major histocompatibility complex (MHC) class II transactivator; FIIND-domain with function to find; IPAF-interleukin 1β-converting enzyme protease-activating factor; LRR-leucine-rich repeat; NACHT-domain present in NAIP; CIITA-HET-E (incompatibility locus protein from Podospora anserine) and telomerase-associated protein; NAD-NACHT-associated domain; NAIP-neuronal apoptosis inhibitor protein; NALP-NACHT domain, LRR domain and pyrin domain-containing protein; PYD-pyrin domain.

The NACHT domain and the LRRs are the only domains that are common for all NLR family members (figure 1). The NACHT domain is responsible for triggering the signaling complex via ATP dependent oligomerization, which is believed to be the crucial step in NLR activation.[3] The LRR domain is comprised of 20-30 amino acids repeats with a characteristic pattern rich in the hydrophobic amino acid leucine. Each structural unit consists of a β−strand and an α-helix that forms a nonglobular, horseshoe-shaped molecule. LRR plays a role in ligand sensing but the mechanism is yet poorly understood.[5,7] Furthermore, the N-terminal domain, which is either a CARD or PYD domain, varies in NLR subfamilies. These N-terminal effector domains are often involved in pathways which activate the transcription factor NF-κB or lead to the activation of caspases.[5,7]

Functionally and structurally, three different NLR subfamilies are distinguished: NALPs or

NLRPs, which represent the largest group, IPAF/NAIP, and NODs. Several NALPs like NALP1, NALP2, and NALP3 were shown to be the central scaffold of caspase-1-activating complexes known as inflammasomes. IPAF/NAIP also play a role in the formation of the inflammasome. [2,3]

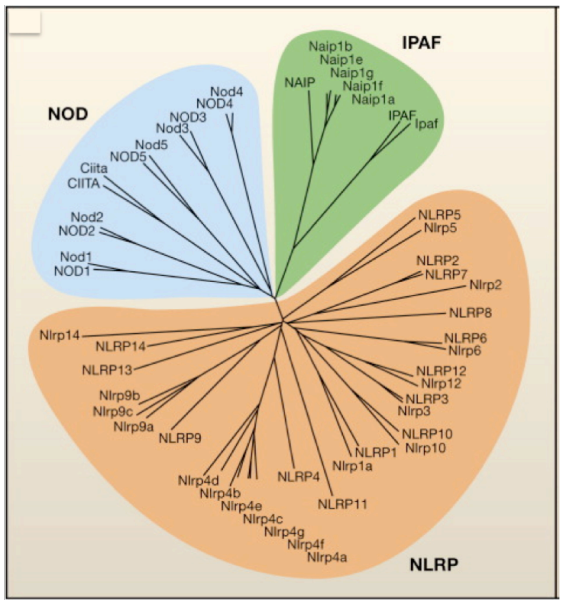

Figure 2 | NLR- subfamilies. Nod-like receptors (NLRs) are divided into three subfamilies: NLRP or NALP, which present the largest group, IPAF and NOD. Figure shows mouse (lower case) and human (upper case) domains. (Picture taken from Schroder *et al* [4])

1.2. The Inflammasomes

Jürg Tschopp and colleagues coined the term "inflammasome". The word is made up of the word inflammation, which referrers to the function of the complex and the suffix "some". "Some", from the Greek word soma, means body, and defines units or molecular complexes such as proteasome, liposome, ribosome, etc. Furthermore the term inflammasome was chosen to point out the structural and functional similarities to the apoptosome, another molecular complex that triggers apoptosis. Functionally, the inflammasomes are high molecular weight complexes which activate inflammatory caspases and lead to maturation of the proinflammatory cytokines IL-1β and IL-18.[4]

1.2.1. Inflammasome types

So far, four different types of inflammasomes have been described. The best characterized is NALP3 (Nlrp3, cryopyrin), which belongs to the NLR family as well as NALP1 (Nlrp1) and IPAF (Nlrc4) inflammasomes. The fourth type is the AIM2 (absent in melanoma 2), a member

of the PYHIN family.[8]

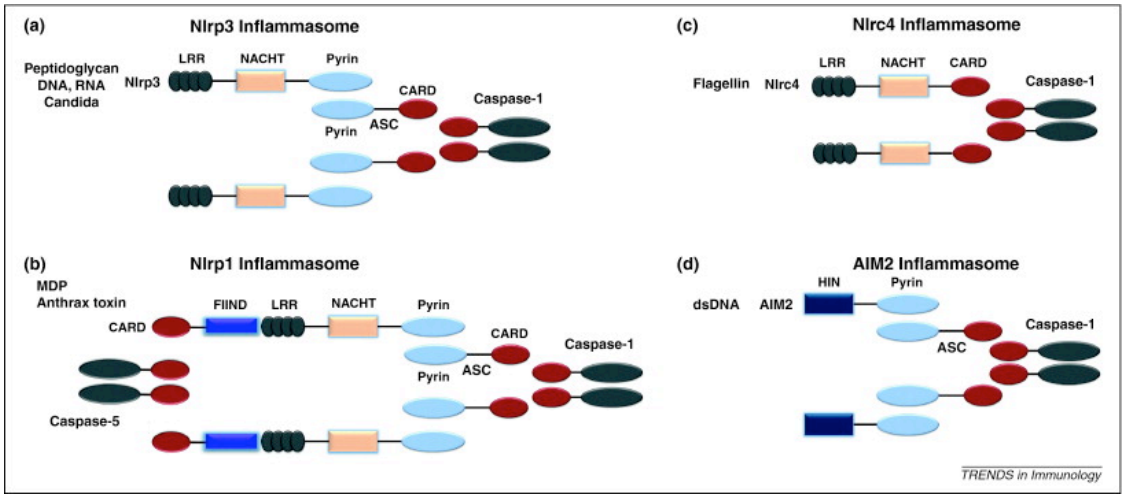

Figure 3 | Schematic representation of the four known inflammasomes. (a) NALP3 inflammasome (b) NALP1 inflammasome (c) IPAF inflammasome (d) AIM2 inflammasome; ASC, apoptosis-associated speck-like protein containing a CARD; FIIND, function to find domain; LRR, leucine-rich repeat; AIM2, absent from melanoma 2; MDP, muramyl dipeptide; picture modified from van de Veerdonk *et al*.[9]

The NALP1 inflammasome

The first described inflammasome was the NALP1 Inflammasome and is up to date the only one reconstituted biochemically. It features some structural differences compared to the NALP3 inflammasome, like a C-terminal FIIND and a CARD domain. NALP1 is the only known type of inflammasome which activates caspase-5 in addition to caspase-1.[8] Activating stimuli of the NALP1 inflammasomes are bacterial ligands such as anthrax toxin or MDP.[10,11] The exact mechanism of how the NALP1 inflammasome is activated remains unclear but K^+ efflux seems to be required.[12,13]

The IPAF inflammasome

Belonging to the NALP family like the NALP1 and NALP3 inflammasome it shares their main structural features like the LRR and NACHT domain. However, the additional N-terminal CARD domain can directly recruit procaspase-1. IPAF activates caspase-1 after recognition of a common motif that occurs in intracellular flaggelin as well as in proteins present in the type III secretion system of the Gram-negative bacteria *Salmonella typhimurium, Burkholderia pseudomallei, Escherichia coli, Shigella flexneri,* and *Pseudomonas aeruginosa.*[4,14] Nevertheless, flaggelin-independent pathways have been described implicating that IPAF can also be activated by other PAMPs.[15,16]

The NALP3 Inflammasome

Up to date the NALP3 inflammasome is the most intensely studied among the different types. It consists of the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD), which connects the NALP with caspase-1. The activation of the NALP3 inflammasome can be due to different kinds of stimuli: PAMPs, whole pathogens, DAMPS (like uric acid crystals or amyloid-β), and also environmental irritants like asbestos.[4,9]

The AIM2 inflammasome

The recently described AIM2 inflammasome, a PYHIN family member, is able to sense cytoplasmic DNA and induces caspase-1 activation. The structure of AIM2 differs from the inflammasomes belonging to the NLR-family. It has a pyrin and DNA-binding HIN domain that forms a complex with ASC and caspase-1.[17] AIM2 binds cytosolic dsDNA of a broad variety of microbial species and also mammalian cells, which may be a link to diseases such as systemic lupus erythematosus that are characterized by autoimmune reactivity against nucleic acids.[18,19]

Although the four types of the inflammasome are activated through different mechanisms and stimuli, their activation always triggers caspase-1 and subsequently the inflammatory cytokines IL-1β and IL-18 are released.

1.2.2. Caspase-1 activation and IL-1β release

Caspases (cysteinyl-aspartate-specific proteinases) have a crucial role in apoptosis, necrosis, and proinflammatory cytokine maturation. 13 different caspases are known in humans, which in turn can be distinguished into the three following groups: executioner (caspase-3, -6, -7) and initiator (caspase-2, -8, -9, -10) of apoptosis, which are both cell death caspases, as well as inflammatory caspases (caspase-12, -11, -5, -4, -1).[20] Concerning the inflammasome, the inflammatory caspase-1 plays an important role. The name inflammatory can be explained due to the main substrates, which are the cytokines IL-1β, IL-18, and possibly IL-33.[8] All of them are important in inflammatory response. Caspase-1 was the first caspase to be discovered in mammals but little was known about how it is activated. The discovery of the inflammasome later shed light on its function.[8]

IL-1β is mainly produced by macrophages, monocytes and dendritic cells, although it can also be secreted by leukocytes in immunologically privileged organs such as brain, kidney, skeletal

muscles, and heart.[21]

IL-1β is the main cause of acute and chronic inflammation and plays a role in the initiation of local and systemic responses to infections, injury, and immunological challenges by activating lymphocytes, chemotactically attracting leukocytes to the site of injury or infection and by generating fever. It has an effect on nearly every cell type either alone or in combination with other cytokines. Extracellularly, IL-1β binds to the IL-1RI receptor, activates a signaling complex, which involves IκB kinase (IKK) and mitogen-activated protein kinase (MAPK) pathways and subsequently triggers the transcription factors nuclear factor (NF)-κB and AP-1. Consequentially, transcription of genes encoding chemokines, acute-phase proteins, cytokines, and cell-adhesion molecules is turned on.[22,23]

IL-18 and IL-33 both have similarities with the IL-1 family proteins. Like IL-1β, IL-18 has an inactive precursor protein which is cleaved by caspase-1. However, inflammatory stimulation has no impact on IL-18 production due to a substantial pool inside the cell.

At least *in vitro*, pro-IL-33 (30 kDA) is as well cleaved by caspase-1 to the mature IL-33 (18 kDA).[22]

1.2.3. Crystals and 'danger' signals trigger NALP3 inflammasome activation

Different signals and stimuli have been identified so far as direct or indirect activators of the NALP3 inflammasome.

Cell disruption was found out to spontaneously activate the inflammasome, which may be due to K^+ efflux. Furthermore, K^+ efflux can be triggered by various stimuli that activate the NALP3 inflammasome. These findings suggest that the inflammasome is able to sense a decrease in potassium levels.[24,25]

Monosodium urate (MSU) as well as calcium pyrophosphate dihydrate (CPPD) crystals, associated with gout and pseudo-gout, are known as endogenous 'danger signals' released from necrotic cells and cause inflammation by activating the adaptive immune response. Findings show that the NALP3 inflammasome is activated via MSU and CPPD crystals resulting in IL-1β and IL-18 production. The involvement of the inflammasome was confirmed by the results that macrophages from mice deficient in ASC, caspase-1 or NALP3 showed impaired IL-1β production. Furthermore, *in vivo* studies with ASC-deficient mice or mice deficient in IL-1R also revealed defective neutrophil influx. Still, the exact molecular mechanism how MSU and CPPD are sensed by the NALP3 inflammasome is not known but it is possible that phagocytosis and the generation of ROS are involved.[26]

Silicia and aluminum salt crystals, aluminum hydroxide, and fibrillar amyloid-β are also sensed by the NALP3 inflammasome and result in activation and IL-1β secretion. Some of these stimuli require crystal phagocytosis and crystal uptake, which leads to destabilization of

the lysosomal membrane and the activation of lysosomal proteases.[27]

Although the NALP3 inflammasome is the best characterized, little is known about the mechanism of its activation. Direct ligand binding is unlikely because the numerous activating compounds have diverse molecular structures. Therefore, three alternative activating mechanisms have been suggested.[4]

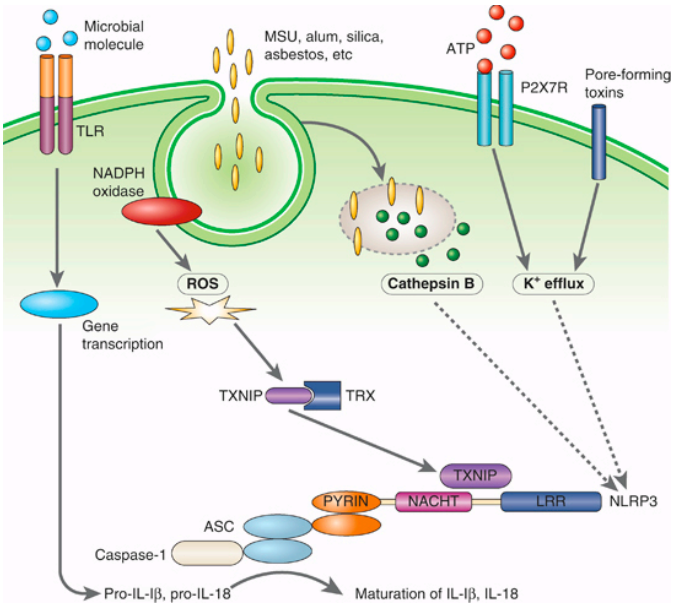

Figure 4 | Different proposed pathways of NALP3 activation. Picture taken from Jin *et al*.[28]

The first mechanism proposes that the stimulation of the purogenic P2X7 ATP ion channel via extracellular ATP leads to K^+ efflux which is needed for triggering NALP3 inflammasome activation.[29] Furthermore, the membrane pore pannexin-1 is recruited, which is required for caspase-1 activation and IL-1β release.[30] Pannexin-1 can act as a specific ATP release channel which hints at an amplifying mechanism for ATP-dependent inflammasome activation, which was in fact studied *in vitro*.[31,32]

The second model indicates that phagocytosis of DAMPs causes rupture and damage in lysosomes and subsequently the release of lysosomal compounds like cathepsin B into the cytoplasm. As a consequence, the NALP3 inflammasome is activated.[27,33]

The third mechanism implicates that inflammasome activation is triggered by reactive oxygen species (ROS). It proposes that ligands that activate the NALP3 inflammasome also stimulate the production of ROS and consequentially the activation of caspase-1.[4]

The three supposed mechanisms may not be exclusive and other pathways for activating the NALP3 inflammasome may exist.

1.2.4. Involvement of ROS in the inflammasome pathway

ROS are free radicals and highly reactive. Structurally, they contain the oxygen atom and include hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) and the hydroxyl radical (OH).[34]

The role of ROS in inflammasome activation is disputed. It was suggested that all NALP3 stimuli share a general pathway leading to the formation of ROS, which subsequently activate the inflammasome.[35] Studies indicate that ROS, in detail NADPH-dependent ROS, is involved and required respectively in NALP3 activation by particle phagocytosis of asbestos or silicia. Moreover, it has been suggested that NALP3 inflammasome activation can be due to excessive ROS production by mitochondria.[4,35]

Recent studies disagreed with these findings. It is claimed that ROS alone is not sufficient to trigger NALP3 activation because LPS leads to ROS production in macrophages although it cannot activate NALP3.[27] Furthermore, cells from patients with chronic granulomatous disease (CGD) who are deficient in NADPH-dependent ROS production showed expected or even elevated levels of IL-1β secretion upon stimulation of caspase-1.[36,37]

Those findings are conflicting. One suggested reason for this could be the use of chemical ROS inhibitors like diphenyleneiodonium chloride (DPI), which indeed inhibit ROS generation, but it also negatively influences the IL-1β transcription leading to reduced IL-1β secretion.[37] Despite all the work that has been done, the role of ROS in NALP3 activation remains to be further elucidated.

1.2.5. Distinction between the inflammasome activation regarding cell types

Studies revealed diverse findings about the activation of the inflammasome in different cell types: *In vitro*, the monocytic cell line THP-1 was stimulated with LPS and no IL-1β release was detected. Consequentially, it was proposed that LPS alone is not sufficient to trigger IL-1β secretion.[38] However, in contrast to the cell line THP-1, IL-1β is released by naturally occurring monocytes when stimulated with a single TLR ligand.[39] It was found that IL-1β production differs between human monocytes and macrophages which shed light on these contradictions.[40] Thus, THP-1 cells, which are often used to study the inflammasome mechanisms *in vitro*, necessitate differentiation of a monocytic cell line into a macrophage-like cell, which can be provoked by e.g. LPS.

Monocytes are able to autocrinely produce ATP that binds the P2X7 receptor and subsequently activates caspase-1 and the inflammasome. Therefore, TLR ligands alone are enough to induce secretion of IL-1β in monocytes and no second stimulus is required. Consequently, monocytes have a constitutive activation of caspase-1. In contrast, the

monocytic cell line THP-1, dendritic cells and macrophages act differently. Two stimuli are needed to induce IL-1β transcription and translation, to trigger caspase-1 activation and subsequently IL-1β secretion (figure 5).[40]

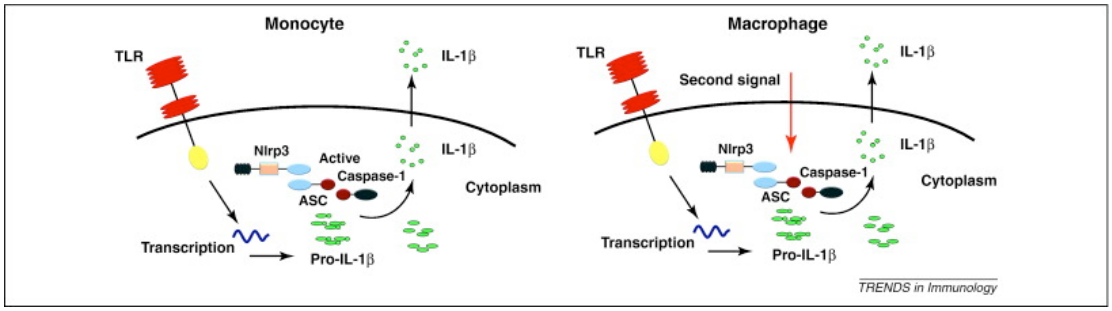

Figure 5 | Different mechanisms of inflammasome activation in monocytes and macrophages. Monocytes have a constitutively active caspase-1 and therefore only need a single stimulus to release IL-1β. In contrast, 2 signals are required to trigger IL-1β release in macrophages; picture taken from van de Veerdonk *et al*.[9]

The difference between the activation mechanism in distinctive cell types is important for the IS. Circulating monocytes have to react without delay to pathogens in contrast to macrophages and dendritic cells. The latter are located e.g. in mucosal surfaces, hence persistently exposed to danger signals and microbial stimuli and the requirement for second signals precludes chronic and harmful reactions by excessive IL-1β release. Nevertheless, during infections elevated ATP levels in the environment operate as a second signal for triggering IL-1β secretion in macrophages and dendritic cells to provide an adequate reaction.[9] LL37, a cathelicidin-derived peptide from infiltrating neutrophils[41] as well as bacterial toxins[42] can also act as a secondary signal.

1.3. Human diseases associated with dysregulation of the inflammasome

Il-1β is important in mediating inflammation and therefore, the release of the cytokine IL-1β (and the related cytokine IL-18) is tightly controlled by at least three distinct steps. First, pro-IL-1β, the 31kDa precursor protein of IL-1β is produced. Pro-IL1β is not constantly present in the cell but induced by pro-inflammatory signals such as TNF or LPS. The second step is the cleavage of pro-IL-1β to the active 17kDa IL-1β, performed by caspase-1 after the activation of the inflammasome. Finally, IL-1β is released in the extracellular environment.[23] The tight control of these steps is crucial since inappropriate release of IL-1β has deleterious consequences such as autoimmune and autoinflammatory diseases.[2]

1.3.1. Cryopyrin-associated periodic syndromes

Gain of function mutations in the NACHT domain of the NALP3 inflammasome are associated with rare autoinflammatory, heritable diseases known as CAPS (cryopyrin-associated periodic syndromes). These diseases are familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and chronic infantile cutaneous neurological articular syndrome/neonatal-onset multisystem inflammatory disease (CINCA/NOMID), differing in their severity but being genetically and phenotypically related. Clinically they are characterized by recurrent fever and inflammation. Isolated macrophages and monocytes from these patients showed spontaneous secretion of IL-1β without any external stimuli and patients respond well to IL-1β antagonists like anakinra.[43]

1.3.2. Gout

Another autoinflammatory disease strongly associated with the NALP3 inflammasome is gout. Gout is characterized by edema and erythema of the joints and neutrophil influx in the intraarticular and periarticular space. The hallmarks of this disease are elevated blood uric acid levels and the accumulation of MSU crystals in joints. As already described, MSU crystals are potent activators of the NALP3 inflammasome *in vitro* as well as *in vivo* models.[26] Blockade of IL-1β has a dramatic effect, which is another proof of the role of the inflammasome activation in disease progression.[44]

1.3.3. Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease characterized by the presence of autoantibodies against nuclear antigens, especially double-stranded DNA. In mouse models for SLE the cytokines IL-1β as well as IL-18 seem to play a role in the progression of the disease[1] and a preliminary study showed the potency of IL-1 blockade with anakinra.[45] Additionally, AIM2 expression is elevated in leukocytes from patients but a correlation between AIM2 expression and SLE disease activity indexes could not be determined.[46] Recent studies have shown that the inflammasome inhibitor bortezumib prevented lupus nephritis in the NZB/W female mouse model[47] and also seems to work in lupus patients that did not respond to other treatments (F. Hiepe, unpublished observations). These findings indicate a potential role of AIM2 or another inflammasome forming complex in the development of SLE.

1.3.4. Rheumatoid arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease, which affects approximately 0.3-1% of the population worldwide. Clinically, it is characterized by persistent synovial inflammation, cartilage degradation and bone erosion. Moreover, a lot of different macrophage and fibroblast cytokines are involved in the pathogenesis, including IL-1, IL-6, IL-15, IL-18, TNF-α, diverse chemokines and others. Although the pathogenesis is still incompletely understood, autoimmunity is assumed to play a critical role. Shown by clinical and experimental studies IL-1β contributes to the pathophysiology of RA and blockade results in reduced signs and symptoms like joint swelling and pain.[48–50] However, the role of the inflammasome in RA in humans is not clear yet and requires further studies.
In murine animal models it was demonstrated that increased IL-1β levels lead to more severe synovial inflammation and cartilage destruction. Both antigen induced arthritis (AIA) and collagen induced arthritis (CIA) share several clinical and pathological features with RA. Investigations of the role of the inflammasome in these models came to the result that disease progression is independent of NALP3 and caspase-1 and, interestingly, an independent function of ASC was concluded.[49,50] Therefore, the NALP3 inflammasome does not seem to play a role in RA mouse models. However, it is not excluded that other inflammasome complexes may contribute to disease progression in mice models.[51]

1.4. Inhibitors of the inflammasome

Uncontrolled action of the inflammasome and especially the NALP3 inflammasome was shown to be associated with disease. Up to date, only IL-1 blockers like anakinra, rilonacept, or canakinumab are used clinically but inflammasome inhibitors may be an effective therapeutic option in the future.[43,52]

Colchicine

Colchicine is an alkaloid extracted from the plant *Colchicum autumnale* also known as meadow saffron or autumn crocus. Interestingly, treatment with colchicine has been used for nearly 4000 years and it was one of the first anti-inflammatory drugs identified. In modern medicine it is still used to treat patients with acute attacks of gout. Recent studies about the efficiency of colchicine in crystal-induced inflammation have shed new light on this topic.[26,53]
Many different biological and pharmacologic effects of colchicine are known. It inhibits microtubule polymerization by binding to tubulin which has an inhibiting effect on many cellular functions like secretion and intracellular vesicle transport. *In vitro*, colchicine reduces

neutrophil chemotaxis and *in vivo* neutrophil adhesion to the vascular endothelium is diminished.[53] In MSU crystal-induced NALP3 inflammasome activation *in vitro*, pre-treatment with colchicine was reported to block IL-1β secretion completely.[26]
However, high toxicity, severe side effect, and potential over dosage make the application of colchicine difficult in humans.

Glibenclamide (Glyburide)

Glibenclamide, belonging to the class of sulfanylurea, is frequently used for the treatment of type 2 diabetes. The drug is known to inhibit ATP-sensitive K^+ channels in pancreatic β-cells. Recently, the effects of glibenclamide on the NALP3 inflammasome were investigated. K^+, as already described earlier, is important for the activation of the inflammasome. By inhibiting K^+ efflux, the maturation of caspase-1 and pro-IL-1β is blocked and, consequentially, the NALP3 inflammasome. A variety of different stimuli were tested and it was claimed that glibenclamide is acting upstream of NALP3 and preventing PAMP- and DAMP- induced IL-1β secretion.[54]

Z-VAD-fmk

The synthetic peptide z-VAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone) is a pan caspase inhibitor and is frequently used *in vitro* for studying apoptosis. Z-VAD-fmk binds irreversibly to the catalytic site of caspase proteases, subsequently inhibiting induction of apoptosis. Inhibition of caspase-1 leads to reduced NALP3 inflammasome activity and can therefore be used for inflammasome studies. Nevertheless, z-VAD-fmk is not applied therapeutically because the FMK leaving group can converse into the highly toxic fluoroacetate *in vivo.*[55]

1.3.6. The connection between cell death and the development of autoimmune diseases

Cell death does not only occur during infection, the IS is always exposed to dead cells during normal tissue turnover. However, some autoimmune diseases are supposed to be associated with an aberrantly increased cell death or with insufficient clearing of dead cells, leading to autoimmunity due to prolonged exposure to endogenous molecules such as nucleic acids, heat-shock proteins, or citrullinated proteins.[56]
Different processes of cell death are known, among them apoptosis (programmed cell death), necrosis, pyroptosis, and NETosis.
Apoptosis is characterized by fragmentation of DNA, cytoplasmic and nuclear condensation,

and membrane changes. The resulting apoptotic bodies with an intact membrane are marked for phagocytosis leading to "clean" cell death without inflammation. In contrast, damage of the plasma membrane and cell lysis is characteristic for necrosis. As a consequence, cytoplasmic contents are released resulting in an inflammatory response.[57]

Pyroptosis is a highly inflammatory cell death and reliant on caspase-1 activity. It causes the formation of plasma membrane pores and the release of proinflammatory cellular content. Morphologically and mechanistically it differs from other forms of cell death and, as opposed to apoptosis, it even enhances inflammation. Caspases, as mentioned earlier, play an important role in cell death. Caspase-3, caspase-6 and caspase-8 are involved in apoptosis while pyroptosis is caspase-1 dependent. Furthermore, processes leading to cell lysis during pyroptosis are also caspase-1 dependent.[58]

Recently, NETosis, i.e. the formation of neutrophil extracellular traps (NETs) has been described as another cell death program.[59] NETosis is a response of neutrophil granulocytes to invading microorganisms and to a variety of pro-inflammatory stimuli such as LPS or TNF. Extracellular fibers mostly composed of DNA, histones and neutrophil proteins such as antimicrobial peptides are released to trap and kill pathogens. Timely clearance of these structures is crucial considering that NET formation is described to be associated with development of autoimmunity against the contents of NETs that can lead to or exacerbate diseases like psoriasis or SLE.[60,61]

Beside the mentioned forms of cell death, other mechanisms are known, such as autophagy or oncosis. Taken together, cell death might play an important role in the development of autoimmune disease and in animal models helping to study the disease.

1.4. Mineral oils

Mineral oils are complex mixtures of straight chains and polycyclic aromatic hydrocarbons. They are mainly composed of alkanes in the range of C_{15} to C_{40}. Mostly, the oils are a byproduct of the fractional distillation of crude oil. Particular mineral oils are known to induce arthritis as well as other autoimmune diseases in genetically susceptible animal models and in exposed humans.

1.4.1. Pristane

Pristane, also known as TMPD (2,6,10,14-tetramethylpentadecane) is an isoprenoid alkane naturally occurring in various plants, many marine organisms like zooplanktonic copepods and algae, and high levels are found in the livers of planktivorous sharks (hence the name pristane). Biosynthetically, pristane is derived from phytol (3,7,11,15-tetramethyl-2-hexadecene-1-ol), an ubiquitous ester of chlorophyll.[62]

1.4.2. Hexadecane

Hexadecane ($C_{16}H_{34}$) is an alkane, which is known to be arthritogenic. However it causes a milder form of RA in DA rats than pristane.[63]

1.4.3. Hexadecene

The mineral oil hexadecene ($C_{16}H_{32}$) is non-arthritogenic although the chemical structure is similar to hexadecane differing in only one additional double binding. [63]

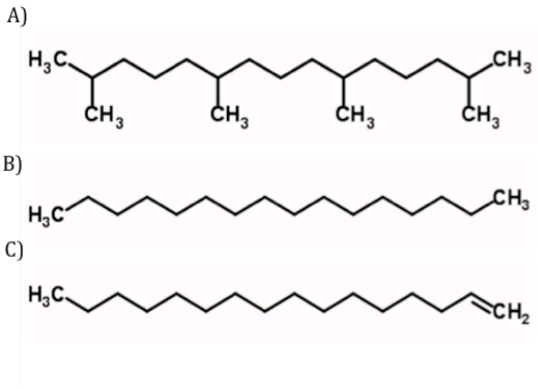

Figure 6 | Chemical structure of mineral oils. A) Pristane B) Hexadecane C) Hexadecene (pictures from www.chemspider.com).

1.4.4. Pristane-induced arthritis

Animal models are helpful for studying the pathogenesis of RA. Many different models are known today, often used examples are collagen-induced arthritis (CIA), oil-induced arthritis (OIA, induced by incomplete Freund´s adjuvant), or pristane induced arthritis (PIA). All these models mimic human RA closely but none of them shares all the characteristics of the disease in humans. In mice it was shown that pristane induces arthritis in susceptible strains, however, also a lupus-like disease, characterized by autoantibodies against DNA and ribonucleprotein, can be caused.[64–66]

In susceptible rat strains, PIA is a well-established model of arthritis since it fulfills many of the clinical criteria like a symmetric involvement of peripheral joints, bone and cartilage destruction, is positive for rheumatoid factor and moreover has a chronic disease course.[65,67]

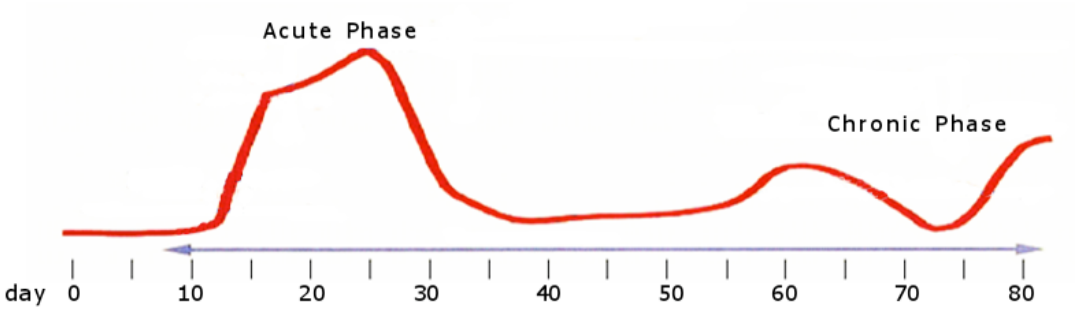

Figure 7 | Disease course typical for PIA in Dark agouti (DA) rats. 150 µl of the mineral oil pristane is injected once subcutaneously or intradermally. The acute phase of arthritis develops after 10 to 14 days and a chronic disease course can be observed 6 to 8 weeks after injection. (picture modified form Holmdahl *et al*.[64]).

Beside pristane, there are other adjuvants like incomplete Freund's adjuvant (IFA), squalene (2,6,10,15,19,23-hexamethyltetracosa-2,6,10,14,18,22-hexaene) and hexadecane which are known to be arthritogenic. Interestingly, a minimal structural difference in hydrocarbons can determine whether the substance induces arthritis or not, e.g. hexadecane ($C_{16}H_{34}$) is arthritiogenic whereas hexadecene ($C_{16}H_{32}$) is not.[63]

To induce PIA, pristane is injected once intradermally or subcutaneously at the base of the tail. The dissemination of oils following injections was shown in a study using ^{14}C-labeled hexadecane in DA rats with a following autoradiography. The arthritogenic oil diffused quickly and was mostly detected in lymph nodes but surprisingly no accumulation was found in the peripheral joints. In other lymphoid organs like spleen and bone marrow, labeled oil was detected as well but more transient than in lymph nodes.[68] The important question, how chemically inert adjuvants like pristane are able to induce arthritis and the molecular mechanism behind, remains unanswered up to now and needs further investigations.

1.4.5. Are Mineral oils a risk factor for autoimmune diseases in humans?

Mineral oils, which are free of unsaturated compounds and aromatics, are used as medicinal, protective coatings for food, and in common commercial cosmetics. Moreover, mineral oils are also found in motor and hydraulic oil.

An *in vivo* study with DA rats tested different commercially available cosmetic products regarding their arthritogenic impact. The products were applied orally, by intradermal injection and, administrated percutaneously, concluding that mineral oils in some cosmetics maintained their adjuvant properties and caused an arthritis-like disease in rats.[69] Furthermore, it was shown that occupational exposure to mineral oils is associated with a

higher risk of developing RA and SLE in men. These studies further suggest a connection between exposure to mineral oil and an autoimmune disease. [70]

2. AIM OF THE STUDY

Animal models have been useful for defining possible mechanisms in the pathogenesis of human autoimmune diseases. Pristane and other mineral oils like IFA and hexadecane are well known adjuvants to induce autoimmune phenotypes, mostly arthritis and lupus-like disease, after injection in experimental animals. Although these animal models are often used to study autoimmune diseases *in vivo*, their way of action is incompletely understood.

The studies performed during this diploma thesis aimed to investigate the effect of pristane and other immunogenic mineral oils on the single-cell level, focusing on induction of cell death and on a possible involvement of the inflammasome in oil-induced autoimmune diseases in animal models.

The effects of different oils (pristane, hexadecane, hexadecene) on the single cell level were tested by stimulating the human monocytic cell line THP-1 as well as freshly isolated splenocytes isolated from DA rats. The tested mineral oils were either applied directly on the cultured cells or as inclusion complexes with β-cyclodextrin to ensure a better administration and easier uptake.

After *in vitro*-stimulation cytokine levels of IL-1β, IL-1α and TNF-α were measured in supernatants. Involvement of the inflammasome in IL-1β secretion was determined by using inhibitors of the inflammasome like z-VAD-fmk or glibenclamide.

Furthermore, flow cytometry was performed to determine morphological changes after treatment with mineral oils and to answer the question whether exposure to mineral oil is connected with increased cell death.

3. MATERIALS AND METHODS

3.1. Buffers and reagents

Unless stated differently, all chemicals were derived from Invitrogen, Gibco, Sigma-Aldrich, or BioRad.

3.1.1. Phosphate buffered saline (PBS), pH 7.2

- 74 mM NaOH • H_2O
- 100 mM NaH_2PO_4
- 1.3 M NaCl

pH 7.2 is adjusted with NaOH, volume is then set to the desired amount with ddH_2O

3.1.2. ACK lysis solution

- 0.15 M NH_4Cl
- 1 mM $KHCO_3$
- 0.1 M EDTA

pH 7.2-7.4 is adjusted, ddH_2O is added to reach a final volume of 1 l

3.1.3. Cell culture – media and growth conditions

- THP-1 cells are cultured with RPMI 1640 (Gibco®) supplemented with 10% FCS, Penicillin/Streptomycin (100 μg/ml) and 10 mM sodium pyruvate.
- D-MEM (Dulbecco's Modified Eagle Medium, Gibco®) supplemented with 10% FCS and 100 μg/ml penicillin/streptomycin is used for culturing rat splenocytes.
- Washing of the cells is done with DPBS, Dulbecco's Phosphate-Buffered Saline, purchased from Gibco®
- Cells are incubated at 37°C in 5% CO_2 unless stated differently.

3.1.4. ELISA coating buffer

PBS with 10% FCS, heat inactivated

3.1.5. ELISA washing buffer

PBS with 0.05% Tween-20

3.1.6. ELISA assay diluent

PBS supplemented with 10% FCS adjusted to pH 7

3.1.7. Stop solution

1 M H_3PO_4 was used as a stop solution

3.1.8. Blocking solution for Western blot

PBS (pH 7.2) supplemented with 0.1% Tween-20 and 5% milk powder

3.1.9. Western blot washing solution

PBS supplemented with 0.1% Tween-20 (PBS-T)

3.1.10. ECL detection reagent

Detection of bands in Western blots was done with Pierce ECL Western Blotting Substrate, purchased from *Thermo scientific*. Solution 1 was mixed with solution 2 1:1 prior to use.

3.1.11. Protein lysis buffer ("Schreiber buffer")

- 20% glycerol
- 0.4 M NaCl
- 1 mM DTT
- 1 mM EDTA
- 1.5 mM $MgCl_2$
- 1 mM EGTA
- 20 mM HEPES, pH 7.9

Protease inhibitor (complete inhibitor cocktail tablets obtained from *Roche*) was added prior to use.

3.1.12. FACS buffer

- PBS with 2% FCS
- 2 mM EDTA

3.2. Cell culture

3.2.1. Isolation of rat splenocytes

Spleens from rats were dissected and kept in sterile PBS. Later on the spleen was pressed through a 40 µm nylon cell strainer with a syringe plunger into chilled PBS to obtain a single-cell suspension. After centrifugation (10 min at 1300 rpm at 4°C), red blood cells were removed by incubating the cell pellet in 2 ml sterile ACK lysis solution for 2 min on ice and filling up with PBS to a total volume of 50 ml thereafter. Subsequently, the cells were centrifuged and washed 2 times with PBS. The resulting pellet was resuspended in medium and the cells were used for further experiments.

3.2.2. Culture of THP-1 cells

THP-1 is a human monocytic cell line first derived from a 1-year old male patient suffering from acute monocytic leukemia. The continuous growth was provided in RPMI 1640 (Gibco®). The cultures were maintained at a density of 2-9 x 10^5 cells/ml at 37°C in 5% CO_2.

Freezing of cells

1x10^6 cells were washed with PBS and after centrifugation (1300 rpm, 10 min) the pellet was resuspended in freezing medium containing 50% FCS, 40% RPMI, and 10% DMSO. The cells were frozen in cryonic tubes and stored at -80°C.

Defrosting of cells

The frozen cells were defrosted quickly with the help of a 37°C warm water bath. Afterwards

they were transferred to a 15 ml tube, which was filled up with PBS and centrifuged at 1300 rpm for 10 min. The pellet was resuspended in 1 ml RPMI and was transferred to a cell culture flask.

Stimulation of THP-1 cells

THP-1 cells were stimulated to increase the phagocytic properties and to induce the production of pro-IL-1β. As a result, the cells switched from being non-adherent to adherent.

a) Stimulation of THP-1 cells with PMA (Phorbol-12-Myristate-13-Acetate):
   THP-1 cells were stimulated for 3 h with 0,5 μM PMA in RPMI. Afterwards the cells were washed gently with PBS, fresh culture medium was added and incubated again at 37°C in 5% CO_2. 24h later the cells were washed again with PBS and ready for further experiments.
b) Stimulation of THP-1 cells with LPS:
   Cells were stimulated for 3h with 1 μg/ml LPS in RPMI. Thereafter cells were washed with PBS and used for further experiments.

3.2.3. Solubilization and complexion of oils

a) Oils bound to FCS
   1 ml pristane was added to 9 ml FCS and rotated for 48h at 4°C as described.[71] Thereafter the surface layer of non-incorporated oil was removed and the lower fraction was used at 10% for cell culture. The final concentration of the incorporated oil was ~ 1 μg/ml, the final concentration in cell culture was therefore 100 ng/ml.
b) Oils complexed to β-cyclodextrin (as described in Janz *et al.*[72])
   4 mM cyclodextrin in H_2O dest. was mixed with mineral oil (final concentration 2 mM) and stirred for 4 days at RT. Afterwards the tube was centrifuged at max speed and the formed complexes were washed 2 times with PBS. After the precipitate was resuspended in 1 ml PBS the concentrations of different oils were adjusted using UV-spectrometry (254 nm). In cell culture the complexes were used 1:200 or 1:150.

3.2.4. Neutrophil Isolation and detection of NETs

For the neutrophil isolation (as described in Russo-Carbolante *et al.*[73]) approx. 3 ml heparinized rat blood was used and carefully pipetted on 3 ml Ficoll Hypaque (*HISTOPAQUE*

1077 mg/ml, Sigma-Aldrich). Afterwards it was spun at 400 g for 45 min without brake (since a gradient was built i.e. cells are in layers).

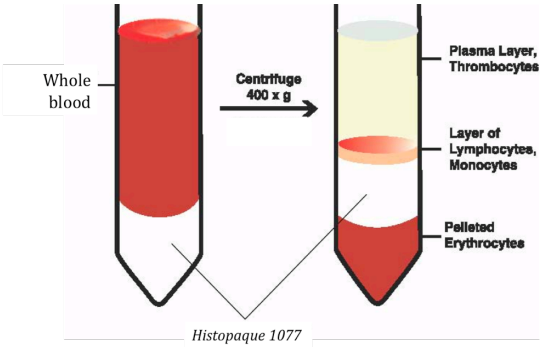

Figure 8 | Histopaque gradient. Heparinized blood was pipetted on 3ml Histopaque 1077 and centrifuged at 400 g. The resultant layers from top to bottom: Plasma – mononuclear cells – Histopaque gradient – red blood cells with neutrophils (picture modified from http://www.himedia.cz/produkty/lymfo).

The upper phase containing plasma was removed and kept for later cultivation of the neutrophils. The neutrophils and red blood cell fraction was transferred into a new tube and 2 ml 6% dextran solution in 0.15 mM NaCl was added. The volume was increased to 4.2 ml with PBS. After incubation for 20 min in a 37°C warm water bath the collected supernatants were spun for 10 minutes at 270 g. The resulting pellet was resuspended in 0.83% NH_4Cl to lyse remaining erythrocytes, incubated for 5 minutes at room temperature and centrifuged at 480 g for 10 min. Cells were washed once with HBSS (Hank´s balanced salt solution, pH 7.2) at 270 g for 8 min and resuspended in 1 ml plasma.

Cell counting

Cells were counted with a Beckman COULTER COUNTER® Cell and Particle Counter.

The isolated neutrophils were now incubated in a 48-well plate with different oils (pristane or hexadecene) complexed to β-cyclodextrin for 0h, 1h, 2h, and 4h. As a positive control PMA- and MSU-stimulated neutrophils were used.

Cytospin

After the given time cells were transferred into Eppendorf tubes and the same amount of formaldehyde (4%) was added to fix the cells.
The glass slides and cardboard filters were placed into appropriate slots in the cytospin, 100 µl of each sample plus 200 µl of PBS were pipetted into the appropriate well and the samples

were spun at 1000 rpm for 6 minutes. The filters were removed carefully and the slides were dried over night.
On the next day the cells were stained with DAPI (4', 6-diamidino-2-phenylindole) and analyzed with the help of a fluorescent microscope.

3.3. Detection of cytokines

ELISA and Western blot analysis were performed starting from supernatant of THP-1 cells ($1x10^6$ cells/well; 48-well plate) or rat cells ($6x10^6$ cells/well; 24-well plate) after o/n incubation with either oils (pristane, hexadecane, hexadecene) complexed to β-cyclodextrin or pristane bound to FCS. As a positive control monosodium urate crystals (InvivoGen), zymosan and LPS was used, olive oil served as a negative control.

3.3.1. ELISA - Enzyme-linked immunosorbent assay

Human IL-1β, IL-1α and TNF-α as well as rat IL-1β, IL-1α and TNF-α secretion was analyzed following the OptEIA™ ELISA Sets (BD Pharmingen) protocol as follows.

ELISA plates (*NUNC maxiSorp*) were pre-coated with a polyclonal antibody diluted in coating buffer (PBS + 10% FCS) specific for the analyzed cytokine and incubated over night at 4°C. The next day the wells were washed 3 times with washing buffer and a blocking step was performed for 1h at room temperature with 200 µl of assay diluent. The plate was washed again three times. Next, 100 µl or 200 µl (dependent on the detected cytokine) of each sample, standard and control were added in duplicates, mixed gently and incubated 2 h at RT covered with an adhesive strip provided. Afterwards, a washing step was performed for five times. Next, 100 µl of the conjugate (biotinylated detection antibody + streptavidin-horseradish peroxidase-conjugate) was added to each well and incubated again for 2 h at RT. The plate was washed 5 times again. Thereafter, 100 µl of substrate solution (tetramethylbenzinide and hydrogen peroxidase) were added and incubated 30 min at RT in the dark. To stop the enzymatic reaction 100 µl of the stop solution was pipetted into each well and mixed gently. Within the next 30 min the optical density was determined by a micro plate reader set to 450 nm with a reference wavelength of 540 nm. The detected cytokine concentration was calculated from a calibration curve performed with standard dilutions.

3.3.2 Western Blot Analysis

Protein extraction

The cells on the 48-well plate were incubated on ice with 150 µl Schreiber buffer (a protease inhibitor cocktail tablet (*Roche*) was added shortly before use (1 tablet for 25 ml of Schreiber buffer)) for 45min; thereafter they were transferred to Eppendorf tubes, sonicated for 5 min and centrifuged for 10 min at 16000 rpm at a table centrifuge. Supernatants were taken and used for Western blot analysis.
To determine the protein concentration of the samples a *NanoDrop*® bioanalyzer was used and the protein concentrations of the samples were adjusted with H_2O dest. before applying them on SDS-PAGE.

SDS PAGE

19.5 µl of the samples were mixed with 7.5 µl 4x SDS sample buffer and 3 µl DTT (0.5 M). Before loading on the gel they were heat-denaturated for 10 minutes at 75°C. After the samples were heated they were loaded (25 µl/slot) on the SDS gel (*NuPAGE® 4-12% Bis-Tris gel*) together with a size marker (3 µl).
The electrophoresis chamber was filled with running buffer and the separation of the proteins occurred at 200 V for 35 min.

Immunoblot

To transfer the proteins from the gel onto the membrane the following procedure was performed:
The "sandwich" *for Bio-Rad's Transblot* was assembled: Sponges, Filter papers, and the membrane (PVDF) were soaked in Transfer buffer. The sandwich was built as sketched in the figure 9.

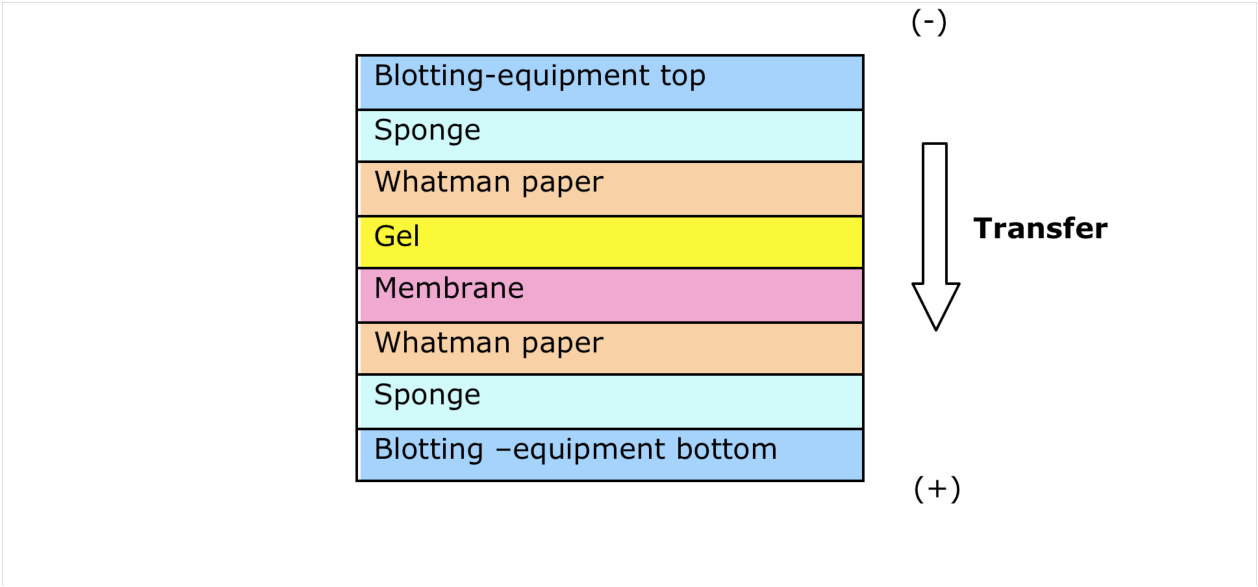

Figure 9 | Schematic assembly of a Western blot

The equipment then was closed and filled up with Transfer buffer (*Bio Rad®*). The blotting was carried out at 165 mA for 1.5 h.

Blocking and Incubation with Antibodies

After successful transfer the membrane was incubated in a solution with PBS + 0.1% TWEEN/5% milk powder for 1 h on a shaker at room temperature to block non-specific binding.
Afterwards, the blocking-solution was exchanged by new blocking-solution containing the proper amount of diluted primary antibody (table 1). Finally the membrane was incubated o/n at 4°C.
Both, before and after the incubation with the secondary antibody (diluted in 5% milk powder in PBS) the membrane was washed for 5 minutes with PBS + 0.1% TWEEN and 2 times with PBS on the shaker at room temperature. The incubation with the secondary antibody took place for one 1h on the shaker at room temperature.

ECL detection

Finally, the detection was done with *Pierce ECL Western Blotting Substrate (Thermo Scientific)*. The membrane was incubated 2 min in 2.5 ml of the prepared ECL solution and excess solution was discarded. The membrane was placed in a transparent sheet, put into a film cassette and an ECL Hyperfilm (Amersham) was exposed to the membrane in a dark room. The exposure time varied between 10 min and 1 h.

Stripping of the membrane and β-actin re-blot

The amount of the protein β-actin was used as a loading control in the western blot analysis. First step was to strip the membrane; the membrane was washed with ddH_2O and incubated for 1 min with 3 ml stripping solution. It was washed again with ddH_2O, then with PBS-T and subsequently the western blot detection protocol was resumed from the blocking step.

For Western blot analysis the following antibodies were used:

Antibody	**Used concentration**	**Company**
Anti rat- Rabbit polyclonal to IL-1 beta	1:1000	*abcam*
Rabbit polyclonal to IL-1 alpha	1:1000	*abcam*
Anti-Human Interleukin-1 beta Polyclonal AB	1:1000	*Thermo scientific*
Anti-Human IL-1β Antibody	1:1000	*Cell signaling*
Anti-Human Cleaved IL-1β (Asp116)	1:1000	*Cell signaling*
ECL Peroxidase labeled Anti-rabbit AB	1:10000	*GE Healthcare*

Table 1 | Antibodies used for Western blot analysis

3.4. Flow cytometry

The cells ($1x10^6$/well) which were used for flow cytometry were incubated on 48-well plates o/n with different oils complexed to β-cyclodextrin. Next, the cells were filtered and transferred to a 96-well plate and were washed twice with FACS buffer at 1500 rpm for 3 min.

3.4.1. Extracellular stain

The cells were resuspended in 200 µl FACS buffer and stained with the appropriate amount of antibody for 30 min at 4°C. Thereafter the cells were washed again 3 times with FACS buffer, resuspended in 200 µl and filtered into FACS tubes.

3.4.2. Intracellular stain

For staining intracellular cytokines the cells were fixed and permebealized. Therefore cells were thoroughly resuspended in 100 µl *BD Cytofix/Cytoperm™ solution* per well and

incubated at 4°C for 15 min. The cells were spun down at 1500 rpm for 3 min, supernatants discarded and resuspended in 50 µl of a saponin-containing buffer (*BD Perm/Wash buffer*) where the intracellular AB was diluted 1:10. After 1 h incubation at 4°C the cells were washed 3 times and the samples were filtered into FACS tubes.

Antibody	***Fluorescent dye***	***Company***
CD54 (mouse Anti-human)	PE	BD Bioscience
CD1a (mouse Anti-human)	APC	BD Bioscience
HLA-DR (mouse Anti-human)	PE	BD Bioscience
CD86 (mouse Anti-human)	APC	BD Bioscience
IL-1β (Anti-human)	PE	BD Bioscience

Table 2 | Antibodies used for flow cytometry

3.4.3. LIVE/DEAD® Cell Stain

The LIVE/DEAD® Fixable Dead cell stain kit from *Invitrogen* evaluated the viability of mammalian cells by flow cytometry. For this purpose $1x10^6$ cells were resuspended in 1ml PBS, 1 µl of the fluorescent reactive dye (corresponding to FL-1) was added and mixed well. After 30 min incubation in the dark the cells were washed twice with FACS buffer, resuspended in 200 µl and filtered into FACS tubes.

3.4.4. AnnexinV/7AAD staining

1 x 10^6 cells per sample were mixed with 1000 µl Annexin V-FITC labeling buffer (ALB) and spun at 1250 rpm for 5 min at 4°C. The supernatants were discarded and the pellet was resuspended in 100 µl ALB and 2,5 µl Annexin V was added to each probe. The tubes were incubated on ice for 20 min. Afterwards 750 µl ALB was added and centrifuged at 1250 rpm for 5 min at 4°C. The supernatants were discarded and the pellets were resuspended with 100 µl ALB + 1:1000 7-Aminoactinomycin (7-AAD).

After the different staining procedures FACS was performed either with Becton Dickson FACSCalibur or FACSCanto. 25.000 events were counted and mathematical correction enabled to compensate for spectral overlap between fluorescent channels. Furthermore, the software FlowJo or Diva was used for data analysis.

3.4.5. Oxidative burst assay of THP-1 cells *in vitro*

THP-1 cells ($1x10^6$ cells/well) were incubated o/n with pristane, hexadecane or hexadecene complexed to β-cyclodextrin. The next day cells were washed twice with PBS and transferred to a 96-well plate in 180 µl PBS.
DHR-123 (dihydrorhodamine-123) in RPMI (3 µM) was prepared and 25 µl of the solution was added to each well using a multi-channel pipette and mixed gently. The incubation took place for 10 min at 37°C. As a control, cells were stimulated with 200 ng/ml PMA in RPMI for 20 min at 37°C.
DHR-123 is a fluorogenic substrate (Fl-1). It determines the percentage of cells that produce reactive oxidants by conversion of DHR 123 to R 123, and their enzymatic activity (amount of R 123/cell).

After washing with PBS the cells were resuspended in 180 µl PBS, filtered in FACS tubes and analysed on a FACSCanto (BD Bioscience). R-123 fluorescence intensity was measured on Fl-1.

3.5. Statistics

Statistical analysis was calculated by one-way ANOVA with the Prism software (v.5; Graph-Pad). Differences were considered significant for a value of $p < 0.05$ and data are shown as mean value ± SEM.

4. RESULTS

4.1. Pilot studies

Pilot experiments were performed to define the most efficient way to solubilize oils and to adapt the concentrations of the complexes formed by different oils. Furthermore, two different ways of differentiating the human monocytic cell line, THP-1, were tried to choose the most effective. All pilot experiments were performed on THP-1 cells. Incubation time was overnight. IL-1β ELISA was used as readout.

Solubilization of oils

Due to insolubility of oils in aqueous medium, two different methods were tested: emulsification of the oil in FCS and complexation to β-cyclodextrin.

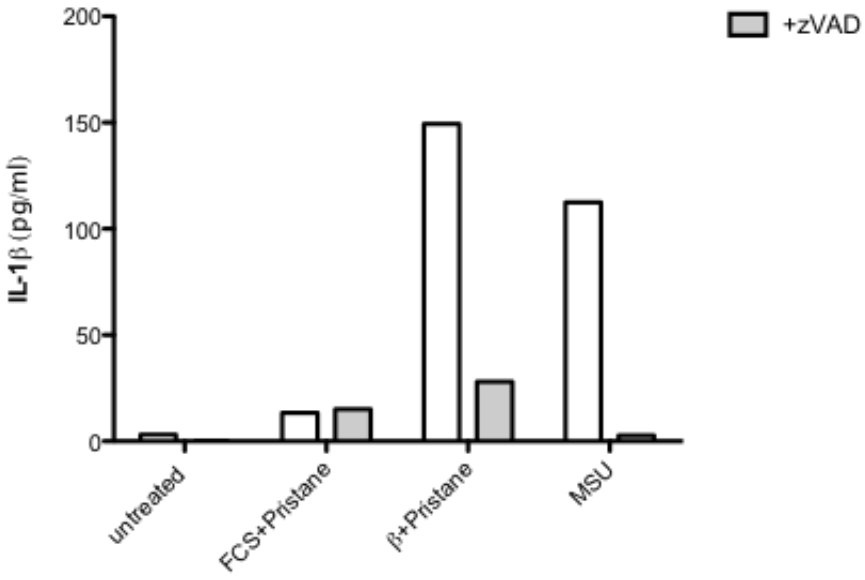

Figure 10 | THP-1 cells treated with pristane bound to FCS, pristane complexed to β-cyclodextrine or MSU crystals. THP-1 cells were differentiated with LPS (1 μg/ml) and incubated o/n either with pristane bound to FCS or inclusion complexes between pristane and β-cyclodextrin (1:200) in the presence or absence of the inflammasome inhibitor z-VAD-fmk. As a positive control, MSU crystals were used. N=1

Mixing the oils with FCS as described in the work of Lee *et al*. was tried to circumvent the problem of the poor immiscibility in aqueous solutions.[71] However, o/n stimulation of THP-1

cells with emulsified pristane in FCS did not show a significantly higher IL-1β production compared to untreated cells. Cells treated with inclusion complexes between β-cyclodextrin and pristane, a method described by Janz *et. al* in 1991[72], upregulated IL-1β production markedly. Hence, complexation was the method of choice (figure 10).

As already described above, THP-1 is a human monocytic cell line, which needs to be differentiated before experimental use. Differentiation can be achieved by various stimuli like PMA (phorbol 12-myristate 13-acetate) or LPS. To determine the best reagent for differentiation, pre-stimulation of the cells was either performed with PMA or LPS (figure 11).

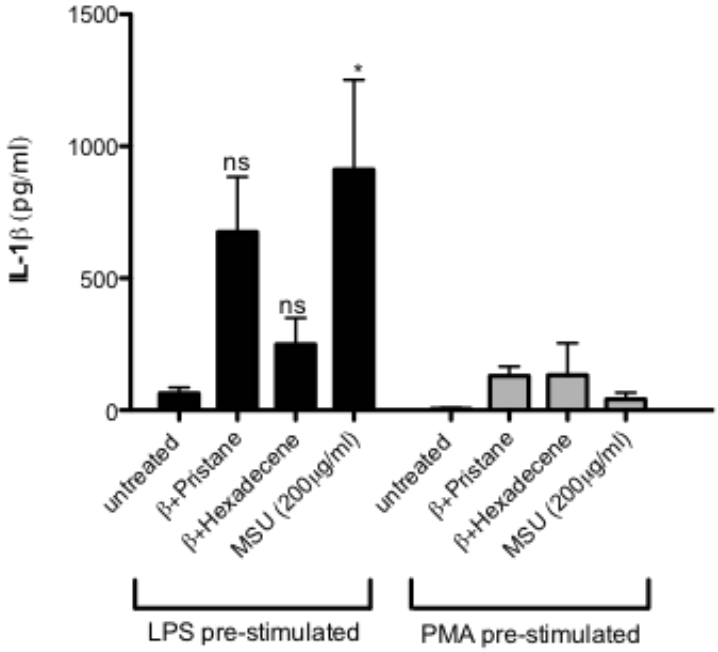

Figure 11 | Pre-stimulation with either LPS or PMA to induce differentiation in THP-1 cells. THP-1 cells were pre-stimulated with 1 µg/ml LPS or 0,5 µM PMA for 3 h. Afterwards, cells were stimulated with indicated oils complexed to β-cyclodextrin (1:200) and incubated o/n. As a positive control MSU crystals were used. Supernatants were analyzed for IL-1β levels by ELISA. Results are shown as mean ± SEM.

After treatment with oils complexed to β-cyclodextrin, supernatants of LPS pre-stimulated cells showed higher IL-1β levels compared to PMA pre-stimulated cells. Consequently, THP-1 cells were pre-stimulated with LPS in further experiments.

Complexes of β-cyclodextrin with different oils (pristane, hexadecane, hexadecene) showed discrepancy in denseness. Thus, concentrations were adjusted with the help of a photometer. Preliminary experiments were done to define an appropriate dilution for using in cell culture

(figure 12).

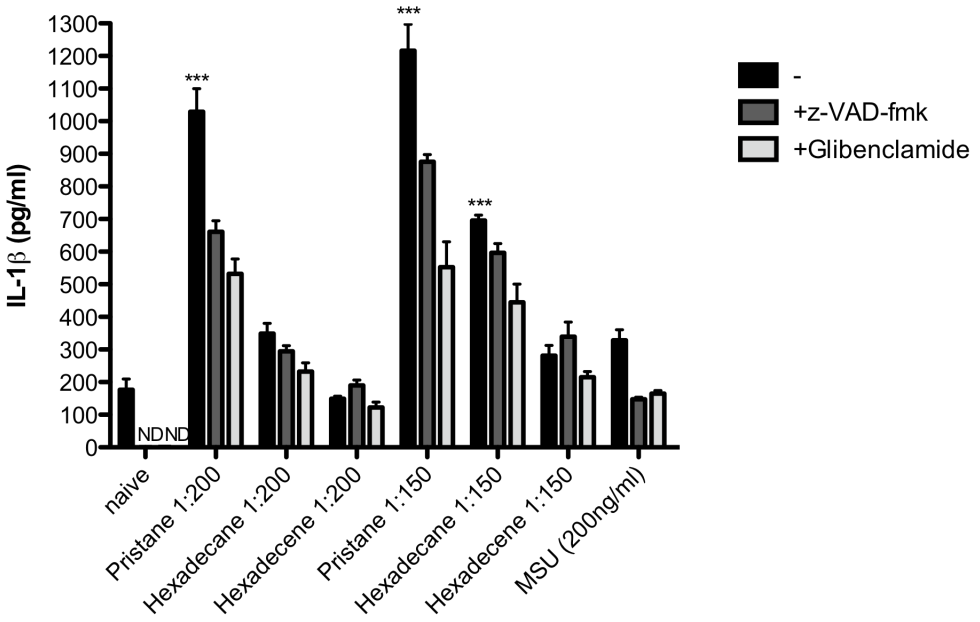

Figure 12 | THP-1 cells stimulated with inclusion complexes of different concentrations. LPS pre-stimulated cells were incubated o/n with pristane, hexadecane or hexadecene complexed to β-cyclodextrin 1:200 or 1:150 in presence or absence of the inhibitors z-VAD-fmk or glibenclamide. MSU crystals were used as a positive control. ND = not done. All results are shown as mean ± SEM.

In figure 12, the differences of IL-1β levels after stimulation with complexed oils of different concentrations are shown. Stimulation with inclusion complexes between β-cyclodextrin and pristane led to significantly higher IL-1β levels regardless of the dilution factor. However, complexes with the arthritogenic oil hexadecane only induced significantly elevated IL-1β levels when used 1:200. Consequently, inclusion complexes were used 1:150 in further experiments.

4.2. Cytokine analysis

4.2.1 *In vivo* analysis

To determine serum cytokine levels during the course of PIA, DA rats were injected once with 150µl pristane intradermally in the tail and arthritis score and different cytokine levels in serum, IL-1β, TGF-β, IL-6, and TNF-α, were determined by ELISA (figure 13).

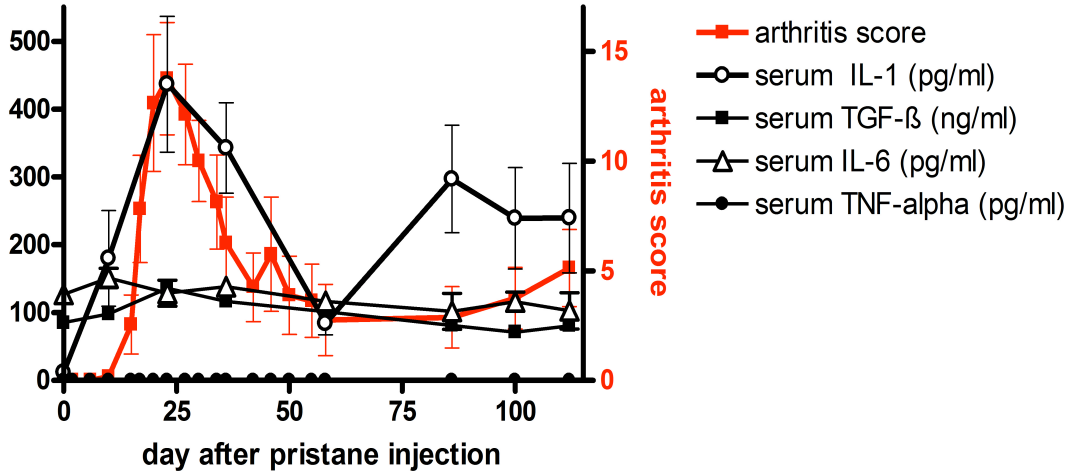

Figure 13 | Serum levels of cytokines during the course of PIA. DA rats were injected once intradermally with pristane and serum levels of indicated cytokines as well as arthritis score were followed over 120 days. Results are shown as mean ± SEM.

Figure 13 shows the disease course of PIA rats. Arthritis developed 12 days after administration of pristane and peaked in severity during acute phase on day 25. Chronic relapses could be observed from 80 days after injection of pristane. Serum levels of the cytokines IL-1β, TGF-β, IL-6 and TNF-α were monitored over the entire disease course and a correlation between disease course and IL-1β could be found. In contrast, TNF-α levels were below detection limits while TGF-β and IL-6 were only slightly increased during the course of disease.

4.2.2. *Ex vivo* and *in vitro* analysis

Conditions for *ex vivo* analysis on rat splenocytes and *in vitro* analysis on THP-1 cells were used as determined in pilot experiments described above.

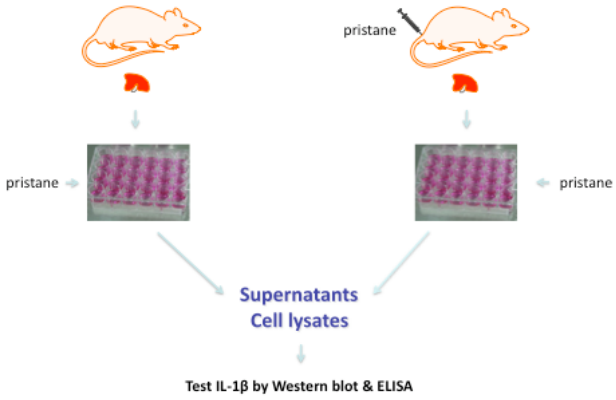

Figure 14 | Schematic demonstration of the procedure. Splenocytes of either naïve or pristane-injected animals were isolated and cultured in 24-well plates. Stimulation with inclusion complexes between β-cyclodextrin and different mineral oils or with pure oils was performed and cells were incubated o/n. Supernatants as well as cells were used for further analysis.

IL-1β secretion

To demonstrate the interplay between *in vivo* and *ex vivo* effects of pristane on IL-1β production rat splenocytes were taken on day 15 and day 21 after pristane-injection and stimulated with pristane complexed to β-cyclodextrin as well as MSU crystals. Inflammasome inhibition was performed with z-VAD-fmk and glibenclamide (figure 15).

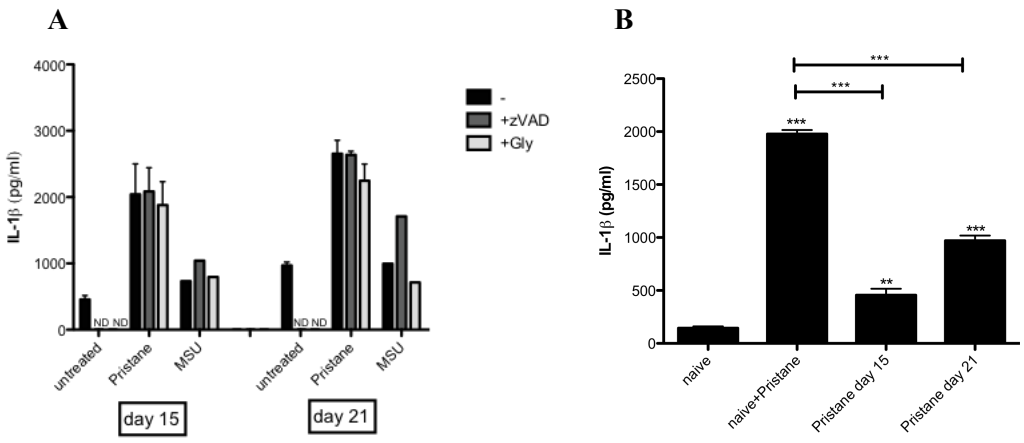

Figure 15 | Comparison of pristane-injected rat splenocytes on day 15 and day 21 regarding IL-1β secretion. A) Splenocytes isolated from PIA rats on day 15 or day 21 were incubated with pristane complexed to β-cyclodextrin or MSU crystals (200 mg/ml) o/n in presence or absence of the inhibitors z-VAD-fmk or glibenclamide. Supernatants were analyzed for IL-1β production by ELISA. B) Comparison between rat splenocytes from naïve animals, isolated splenocytes incubated with pristane and splenocytes taken from PIA rats on day 15 or day 21. IL-1β production was monitored by ELISA. ND = not done. Values are shown as mean ± SEM.

IL-1β levels in untreated cells were higher on day 21 compared to day 15. After additional stimulation with inclusion complexes of β-cyclodextrin with pristane, elevated IL-1β levels could be observed in cells taken on day 15 as well as on day 21. Inhibition with both z-VAD-fmk and glibenclamide did not show a significant reduction of IL-1β levels. Treatment with MSU crystals did not have a large effect on the production of IL-1β (figure 15A).
Figure 15B shows that the production of IL-1β was significantly higher in untreated splenocytes of PIA rats on day 15 and even more elevated on day 21 compared to splenocytes taken from naïve animals. *Ex vivo* incubation with inclusion complexes between β-cyclodextrin and pristane elicited even higher levels of IL-1β than exposure to pristane *in vivo*.

Previous experiments had shown that pristane-priming *in vivo* as well as application *ex vivo* had an impact on IL-1β levels. Therefore, the effect of pristane, hexadecane or hexadecene complexed to β-cyclodextrin *ex vivo* was further investigated. MSU crystals (200 µg/ml) served as a control.

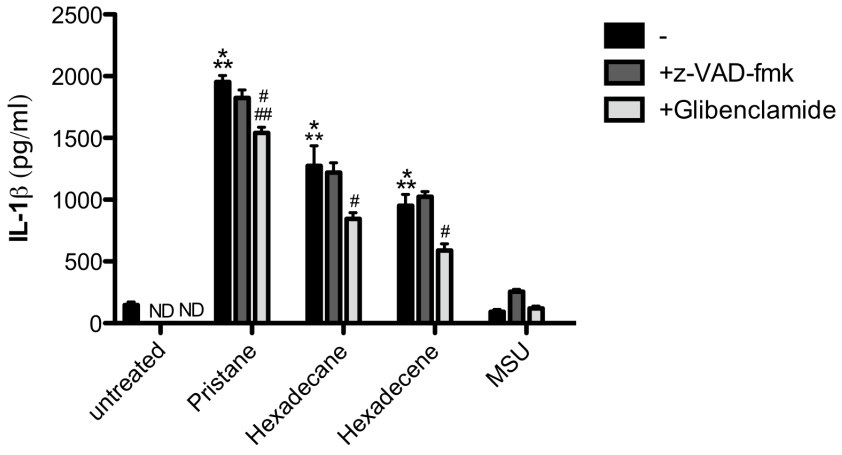

Figure 16 | Stimulation of splenocytes from naïve rats with oils complexed to β-cyclodextrin. Splenocytes of naïve rats were incubated with inclusion complexes between β-cyclodextrin and pristane, hexadecane or hexadecene or with MSU crystals o/n in presence or absence of the inhibitors z-VAD-fmk or glibenclamide. IL-1β levels in supernatants were tested by ELISA. ND=not done. Results are shown as mean ± SEM.

In figure 16, IL-1β production from stimulated rat splenocytes is shown. Stimulation of cells with complexed oils, pristane, hexadecane and hexadecene, led to significantly higher IL-1β levels in comparison to untreated cells. Incubation with MSU crystals showed no significant changes in IL-1β production. Inhibitory effects could not be observed after treatment with z-

VAD-fmk, whereas glibenclamide significantly reduced IL-1β production in splenocytes incubated with pristane, hexadecane or hexadecene complexed to β-cyclodextrin.

Next, the effect of pristane and other mineral oils was tested *in vitro* on THP-1 cells. Cells were differentiated with LPS (1 μg/ml) for 3 h before o/n stimulation with oil-β-cyclodextrin complexes took place in presence or absence of the inflammasome inhibitors z-VAD-fmk or glibenclamide. MSU crystals served as a positive control. Supernatants were taken and analyzed for different cytokines by ELISA.

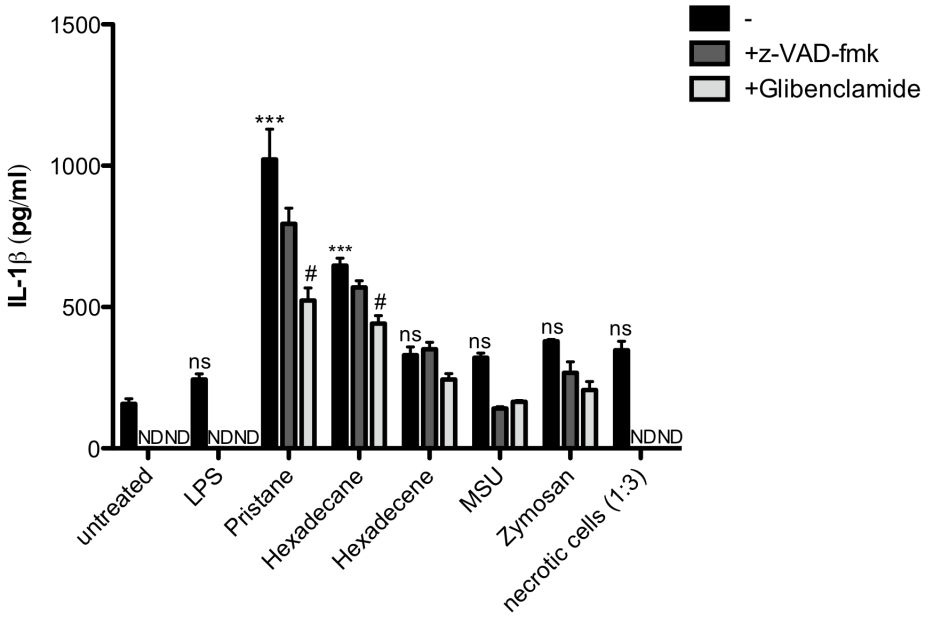

Figure 17 | IL-1β levels after stimulation of THP-1 cells. Differentiated THP-1 cells were incubated o/n with LPS, inclusion complexes between β-cyclodextrin and indicated oils, MSU crystals, zymosan or heat-induced necrotic THP-1 cells in presence or absence of the inhibitors z-VAD-fmk (10 μg/ml) or glibenclamide (25 μg/ml). Supernatants were analyzed for levels of IL-1β by ELISA. ND=not done. Results are shown as mean ± SEM. N=11 for untreated, pristane, hexadecane, hexadecene, and MSU groups: N=3 for zymosan and necrotic cells groups.

Zymosan was used to investigate inflammasome-independent IL-1β release from THP-1 cells because it is known that zymosan activates TLR2 2 and not the inflammasome. Furthermore, it was assumed that necrotic cells might activate the inflammasome and therefore the effect of cells in which necrosis had been induced by heat was tested.

Treatment with pristane and hexadecane complexes elevated IL-1β levels significantly, whereas no significant differences of IL-1β in the supernatants were detected after stimulation with LPS, hexadecene complexed to β-cyclodextrin, MSU crystals, zymosan and heat-induced necrotic cells. Inhibition of pristane and hexadecane stimulated cells with z-VAD-

fmk showed slightly, but not significantly reduced IL-1β levels while a significant decrease could be observed after inhibition with glibenclamide (figure 17).

Molecular encapsulation of oils into β-cyclodextrin seems to be a sufficient method for delivering alkanes to mammalian cells. Nevertheless, the *in vivo* situation is different: cells are confronted with pure oils. Therefore, also the effect of pure oils was tested on isolated rat splenocytes and THP-1 cells.

The optimal time for direct stimulation was determined (on THP-1 cells) as shown in figure 18.

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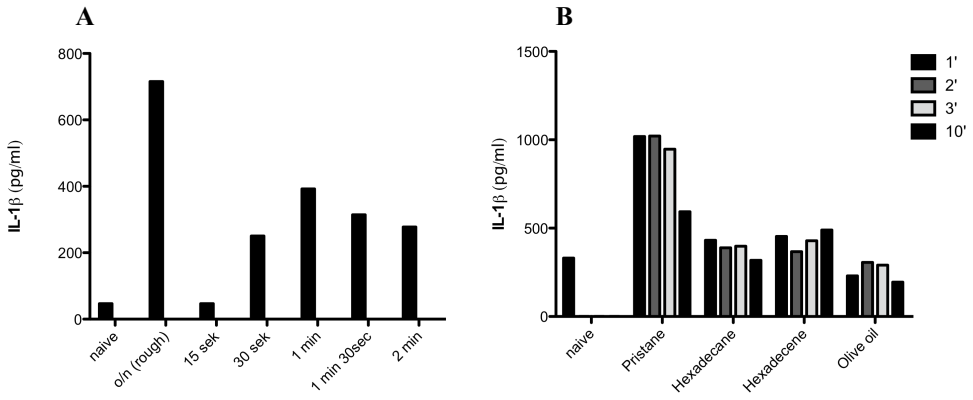

Figure 18 | Determination of the optimal time for stimulation of THP-1 cells with pure oils. THP-1 cells were differentiated 3h with LPS (1 μg/ml) and (A) either stimulated for the indicated time with pristane and cultured o/n in RPMI or pristane-containing medium was mixed roughly with the cells and incubation took place o/n. (B) THP-1 cells were stimulated with pristane, hexadecane, hexadecene, or olive oil for the indicated time and cultured o/n in RPMI. ELISA was performed to analyze IL-1β levels in supernatants. Results are shown as mean ± SEM.

Referring to the data shown in figure 18A, the highest IL-1β levels were observed after mixing pristane-containing medium (100 μl oil and 200 μl medium) roughly with the cells by pipetting up and down quickly and incubating with the oil o/n. However, the incubation time for further experiments was chosen to be 1 minute after microscopic investigations, which indicated approximately more than 70% dead cells when using the o/n method.

Afterwards, the effect of pure oils on THP-1 cells with the optimized application protocol was investigated. Therefore cells were differentiated with LPS (1 μg/ml) for 3h. Thereafter, a 2h pre-incubation with the inhibitors z-VAD-fmk (50 μg/ml) and glibenclamide (125 μg/ml) was performed and oils were put directly on the cells. Stimulated cells were incubated in presence or absence of the inhibitors (figure 19).

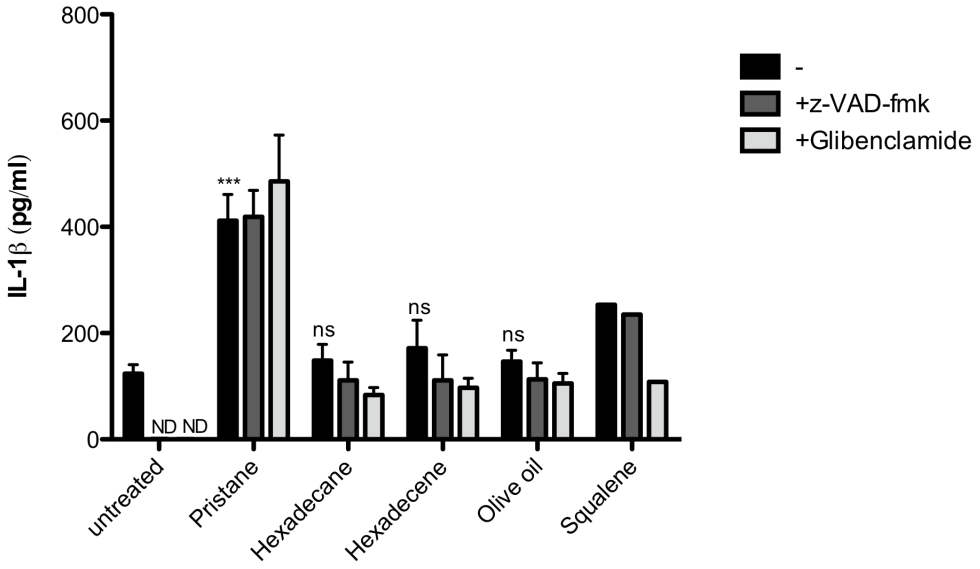

Figure 19 | Treatment of THP-1 cells with mineral oils directly. Differentiated THP-1 cells were pre-incubated with the inhibitors z-VAD-fmk or glibenclamide and stimulated with the indicated oils for 1min. Overnight incubation took place in presence or absence of inhibitors. ELISA was performed to determine IL-1β levels in supernatants. ND=not done. Results are shown as mean ± SEM.

THP-cells stimulated with pristane showed a significantly elevated IL-β production compared to untreated cells. Hexadecane, hexadecene and the control oil olive oil showed no significantly higher IL-1β levels. After stimulation with squalene, another mineral oil known to be arthritogenic[74], higher IL-1β levels were observed but due to small sample number (n=1), statistical analysis could not be done. Slight inhibitory effects of both inhibitors were observed in hexadecane-, hexadecene- and olive oil treated cells and glibenclamide showed inhibition on cells stimulated with squalene (figure 19).

The effect of pure oils was also tested on freshly isolated rat splenocytes. Therefore, the cells were also pre-incubated for 2h with the inhibitors z-VAD-fmk (50 μg/ml) and glibenclamide (125 μg/ml) and stimulated directly for 1min with pristane, hexadecane, hexadecene, squalene or olive oil. Stimulated cells were incubated in presence or absence of the inhibitors (figure 20).

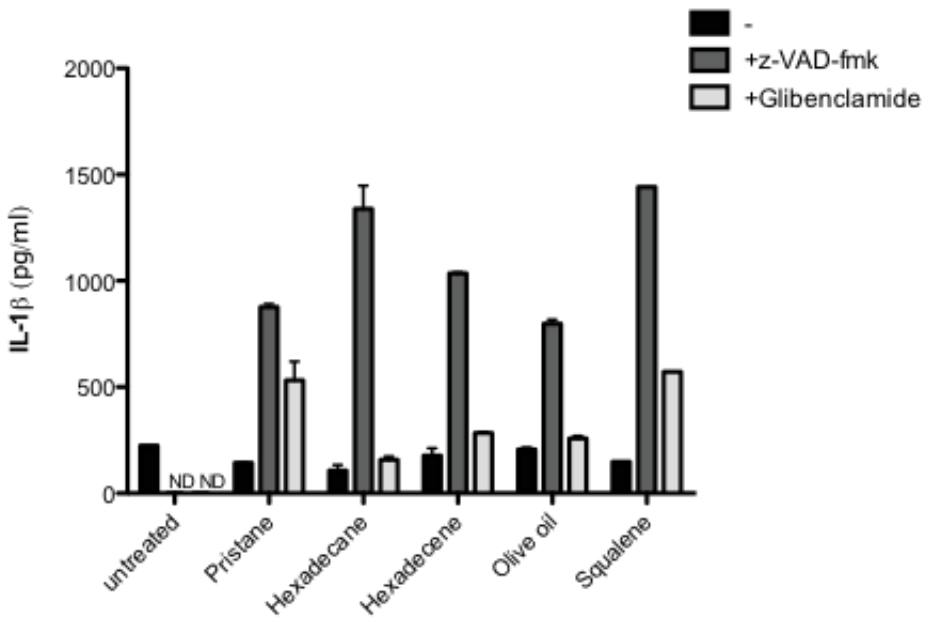

Figure 20 | Effect of pure oils on isolated rat splenocytes. Pre-treated rat splenocytes with the inhibitors z-VAD-fmk or glibenclamide or untreated cells were stimulated with pristane, hexadecane, hexadecene, olive oil or squalene for 1 min and incubated o/n in presence or absence of z-VAD-fmk or glibenclamide. IL-1β ELISA was performed with supernatants. ND=not done. Results are shown as mean ± SEM.

Effect of pure oils and inhibitory effects of z-VAD-fmk and glibenclamide were tested as shown in figure 20. IL-1β levels were not elevated when treated with any stimulus compared to untreated cells. However, pre-incubation with z-VAD-fmk and following stimulation with pristane, hexadecane, hexadecene, olive oil or squalene induced an increase of IL-1β production compared to untreated cells and not pre-incubated cells. Glibenclamide showed slightly higher IL-1β levels after stimulation with pristane and squalene but not upon stimulation with hexadecane, hexadecene, or olive oil.

TNF-α and IL-1α secretion

The release of the cytokines IL-1α and TNF-α upon stimulation of THP-1 cells and rat splenocytes with mineral oils complexed to β-cyclodextrin was measured to investigate inflammasome-independent activities of the oils because they do not depend on inflammasome-activation. Furthermore, zymosan served as a control for inhibition. Zymosan is a TLR2 agonist and inflammasome-independent, thus inflammasome inhibitors should not have an effect on IL-1α or TNFα production upon incubation with zymosan.

In vitro analysis on THP-1 cells regarding IL-1α and TNF-α production are shown in figure 21:

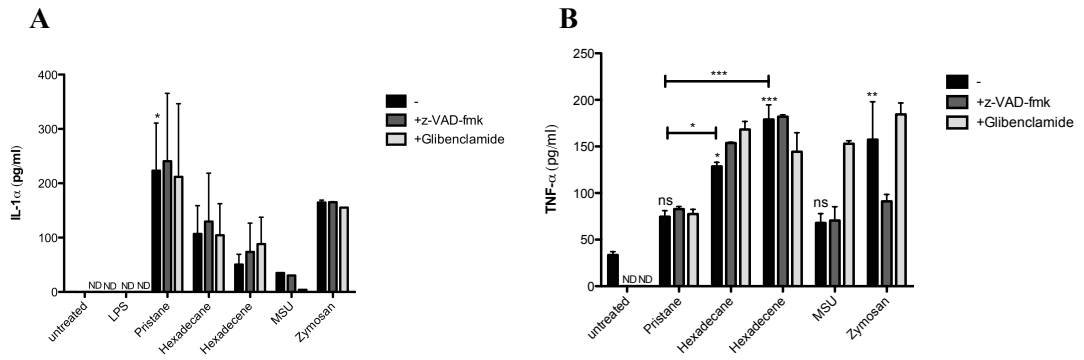

Figure 21 | IL-1α and TNF-α levels in supernatants of THP-1 cells. After differentiation of cells with LPS (1 μg/ml), cells were left untreated or stimulated with LPS, MSU crystals, zymosan or pristane, hexadecane or hexadecene complexed to β-cyclodextrin in presence or absence of the inhibitors z-VAD-fmk or glibenclamide. After o/n incubation supernatants were analyzed for IL-1α (A) and TNF-α (B) levels. ND=not done. Results are shown as mean ± SEM.

IL-1α levels were zero in untreated and LPS stimulated THP-1 cells. Generally, stimulation with complexed oils led to elevated IL-1α levels though differences can be observed between the oils. Stimulation with pristane provoked the highest increase of IL-1α, whereas hexadecane and hexadecene stimulation led to slightly reduced levels compared to pristane. MSU crystals did not show a strong effect on IL-1α production, while incubation with the TLR2 antagonist zymosan elevated IL-1α production. An inhibitory effect of either z-VAD-fmk or glibenclamide could not be observed with the exception of glibenclamide inhibiting MSU treated cells (figure 21A).

Interestingly, TNF-α levels were significantly higher in supernatants of hexadecane and hexadecene stimulated THP-1 cells but not in pristane treated cells compared to untreated cells. Zymosan also led to a significant increase of TNF-α production whereas MSU crystals did not. Inhibition was successful with z-VAD-fmk in zymosan-stimulated cells and glibenclamide had a slight inhibitory effect on hexadecene treated cells (figure 21B).

Next, IL-1α and TNF-α production was also analyzed upon stimulation of rat splenocytes with complexed mineral oils (figure 22).

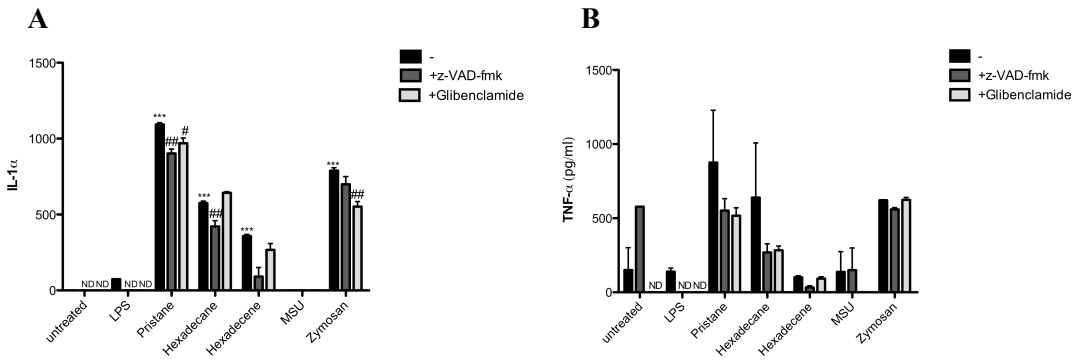

Figure 22 | IL-1α and TNF-α levels after stimulation of rat splenocytes with inclusion complexes between β-cyclodextrin and indicated oils. Supernatants of stimulated splenocytes were tested by ELISA regarding their A) IL-1α and B) TNF-α levels. Stimuli were LPS, oils (pristane, hexadecane, or hexadecene) complexed to β-cyclodextrin, MSU crystals, and zymosan. Incubation was done o/n in the presence or absence of z-VAD-fmk or glibenclamid. ND=not done. Results are shown as mean ± SEM.

IL-1α levels of rat splenocytes after o/n incubation with different stimuli are shown in figure 22A. Compared to untreated cells, which appeared to have no IL-1α production (or release), stimulation with inclusion complexes between β-cyclodextrin and pristane, hexadecane, or hexadecene as well as treatment with zymosan induced significantly higher levels. MSU crystals did not induce any IL-α production while LPS provoked a minimal increase of IL-α levels. Inhibition with z-VAD-fmk reduced IL-1α levels in cells stimulated with complexes significantly while inhibiting effects of glibenclamide were only observed in splenocytes incubated with pristane. Furthermore, elevated IL-1α levels in zymosan treated cells could be decreased slightly with z-VAD-fmk and significantly with glibenclamide (figure 22A).
Pristane and hexadecane complexed to β-cyclodextrin elevated TNF-α levels of rat splenocytes compared to untreated cells and inhibition could be observed by treatment with z-VAD-fmk and glibenclamide. Hexadecene complexes did not show any effect on TNF-α production and also MSU crystals and LPS did not lead to an increase of TNF-α. The treatment with zymosan induced higher levels compared to untreated cells. However, there was no inhibition by z-VAD-fmk or glibenclamide. Interestingly, incubation of untreated cells with z-VAD-fmk increased TNF-α levels instead of decrease (figure 22B).

Western blot analysis

To verify the results obtained, Western blot analysis was performed with THP-1 cells and rat splenocytes (not shown) after stimulation with inclusion complexes between β-cyclodextrin and pristane, hexadecane or hexadecene. The used IL-1β antibodies are able to differentiate

between the precursor of IL-1β (pro-IL1β ~30.7 kDA) and the mature form (IL-1β ~17.5 kDA), which could not be reliably distinguished by ELISA.

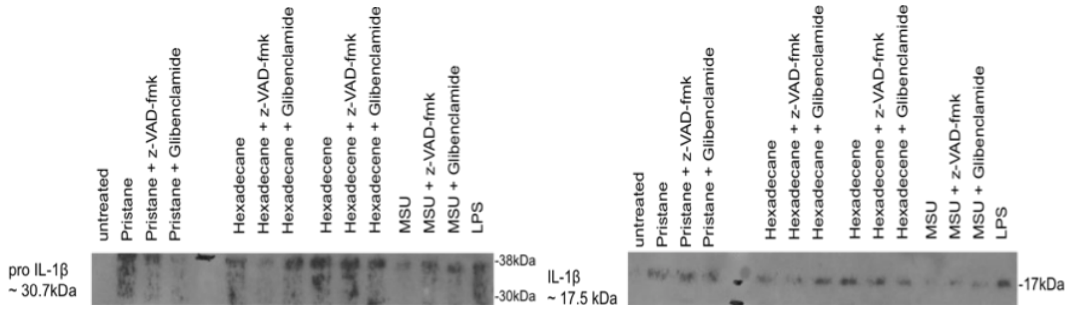

Figure 23 | Western blot THP-1 cells. Western blot was performed after o/n incubation of differentiated THP-1 cells with indicated stimuli in presence or absence of the inhibitors z-VAD-fmk or glibenclamide. Cells were analyzed for the presence of pro-IL-1β (30.7kDa) and mature IL-1β (17.5 kDa).

Western blot analysis was performed several times due to technically non-satisfying results. In most of the cases, no or only very weak bands could be seen on the blots. Figure 23 represents the best obtained results. However, bands are still smeared and unclear especially on the height of pro IL-1β, hence interpretation is difficult.

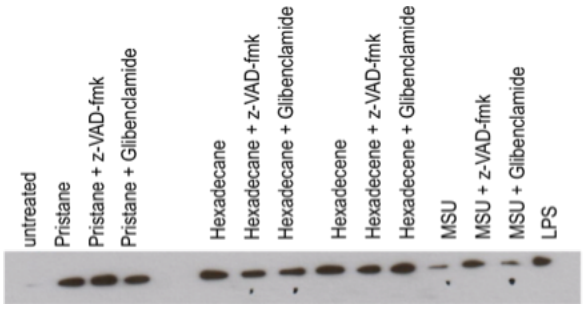

Figure 24 | Actin re-blot. Actin re-blot was done with the membrane shown in figure 23 to determine the loaded protein amounts.

Actin-re blot was done to determine the correct amount of loaded proteins (figure 24). Referring to the blot in figure 23, it was observed that the loaded amounts were too little in the following samples: untreated, MSU, and MSU + glibenclamide. After recalculation Western blot was performed again with the appropriate amounts but no bands could be seen after staining with antibodies to IL-1.

We tried to solve the technical problems by changing the used antibodies (both the first and the second). Moreover, different protocols for cell lysis and Western blot development were used and reagents and buffers were exchanged. Despite all efforts, no sufficient results were achieved.

4.3. Morphological analysis of stimulated cells

The alteration of naïve rat splenocytes and THP-1 cells regarding size, complexity and granularity upon stimulation was determined by flow cytometry.

Figure 25 shows dot plots of rat splenocytes stimulated with inclusion complexes between β-cyclodextrin and pristane or hexadecane. Furthermore, LPS-treated, PMA-treated and unstimulated cells are depicted.

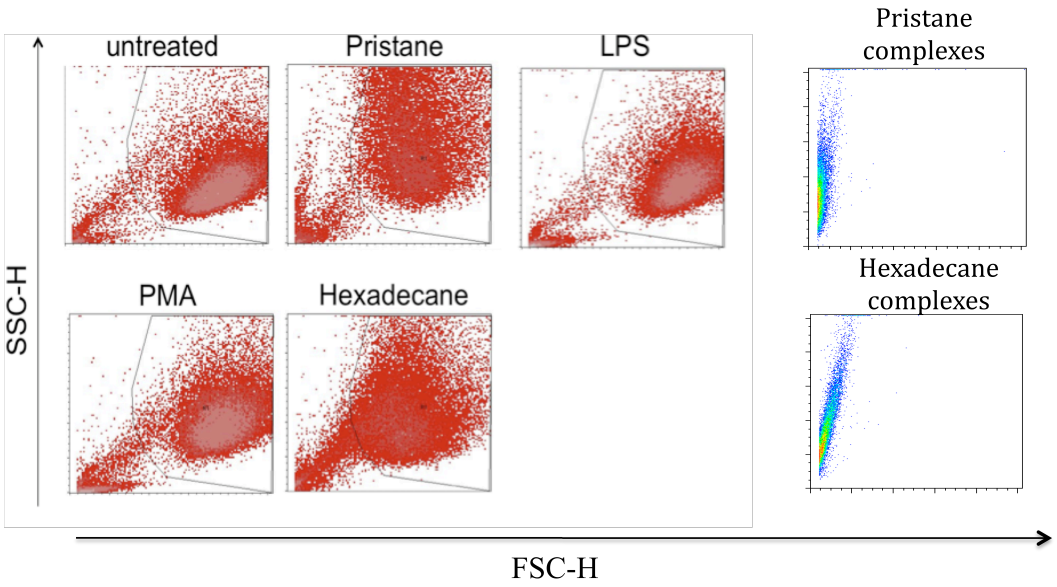

Figure 25 | Dot plots showing naïve rat splenocytes upon stimulation and pristane and hexadecane complexes. Isolated rat splenocytes were incubated with indicated stimuli, cells were left unstained and flow cytometry was performed with a BD FACSCalibur. P1 gate included all viable cells. Pristane and hexadecane complexes alone were analyzed as well by flow cytometry. Analysis was done with Diva software.

The morphology of the cells changed upon stimulation with complexed mineral oils compared to untreated cells. Changes involved an increase of cell granularity and complexity, seen by an increase in SSC. Stimulation with LPS or PMA showed a slight change of the phenotype regarding granularity and size (figure 25). To be sure that complexes alone are not mixed up with cells in FACS blots, FACS analysis of pristane and hexadecane complexes was performed. As seen in figure 25, complexes are not in the gated area of analyzed cells.

The same morphological analyses were done *in vitro* with THP-1 cells (figure 26):

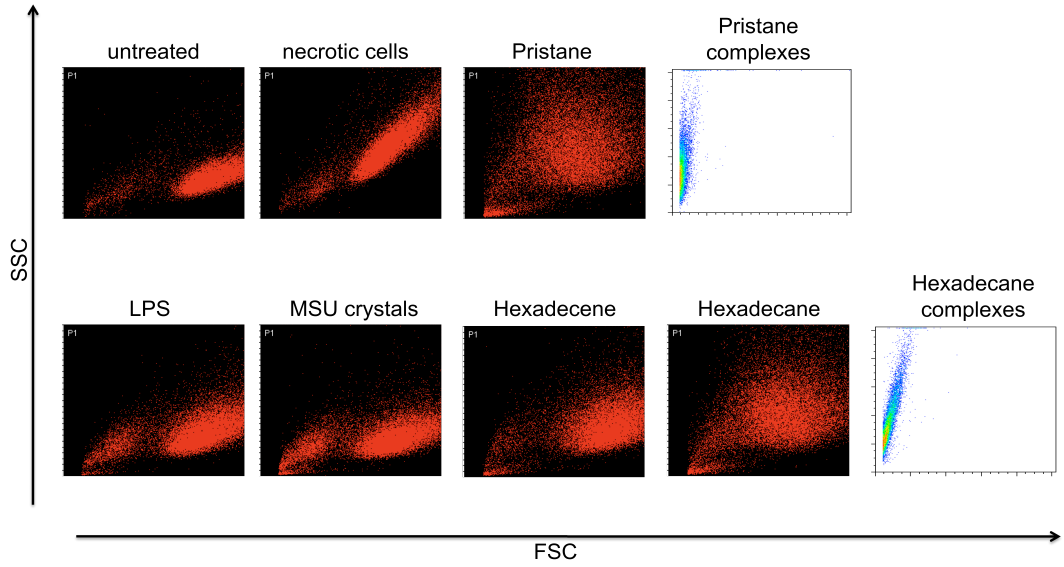

Figure 26 | Dot plots from unstained, stimulated THP-1 cells and pristane and hexadecane complexes. Differentiated THP-1 cells were incubated o/n with LPS, MSU crystals or pristane, hexadecane or hexadecene complexed to β-cyclodextrin; necrosis was induced by heat (56°C, 30'). Cells were left unstained and flow cytometry was performed with BD FACSCalibur. Furthermore, complexes alone, pristane complexes and hexadecane complexes and analyzed as well with flow cytometry. No gates were set to analyze all cells, viable and dead. Following analysis was done with Diva software.

Incubation of THP-1 cells with pristane complexes significantly increased their side scatter (SSC) reflecting increased granularity (figure 26). The same effect could be observed upon treatment with hexadecane. Hexadecene also increased granularity but to a lesser extent than pristane and hexadecane. MSU crystals, LPS and heat-induced necrosis also led to changes in morphology depicted in FSC and SSC compared to untreated cells, but granularity increase was less than upon treatment with pristane or hexadecane complexes. Interestingly, the FSC, reflecting the size, decreased upon incubation compared to untreated cells.

	FSC (mean)	**SSC (mean)**
untreated	224.440	92.461
Pristane	158.793	132.281
Hexadecane	167.136	115.515
Hexadecene	206.234	97.443

Table 3 | Comparison of the FSC (mean) and SSC (mean).

Again, to distinguish between complexes and cells, flow cytometry of complexes alone was done and compared. Dot plots of pristane and hexadecane treated cells as well as pristane and hexadecane complexes alone are shown in figure 26. These comparisons made it possible to

distinguish between complexes and cells in FCS/SSC-blots.

4.4. Apoptosis and cell death analysis upon stimulation

Next, the role of mineral oils in conjunction with apoptosis and necrosis was examined.

4.4.1. 7AAD/AnnexinV staining on rat splenocytes

First, analysis was performed on stimulated rat splenocytes as shown in figure 27 and 28: 7AAD and annexinV staining was performed after cells were stimulated with pristane, hexadecane, or hexadecene complexed to β-cyclodextrin. AnnexinV binds to phospahatidylserine (PS), which is translocated from the cytosolic to the outer surface of the cell membrane in apoptotic cells. 7-amino-actinomycin D (7AAD) intercalates into double-stranded nucleic acids and is therefore excluded by viable cells but can penetrate cell membranes of dying or dead cells.

As controls, cells were left untreated, stimulated with MSU crystals or LPS (1 μg/ml). Flow cytometry was performed with FACSCalibur (figure 27).

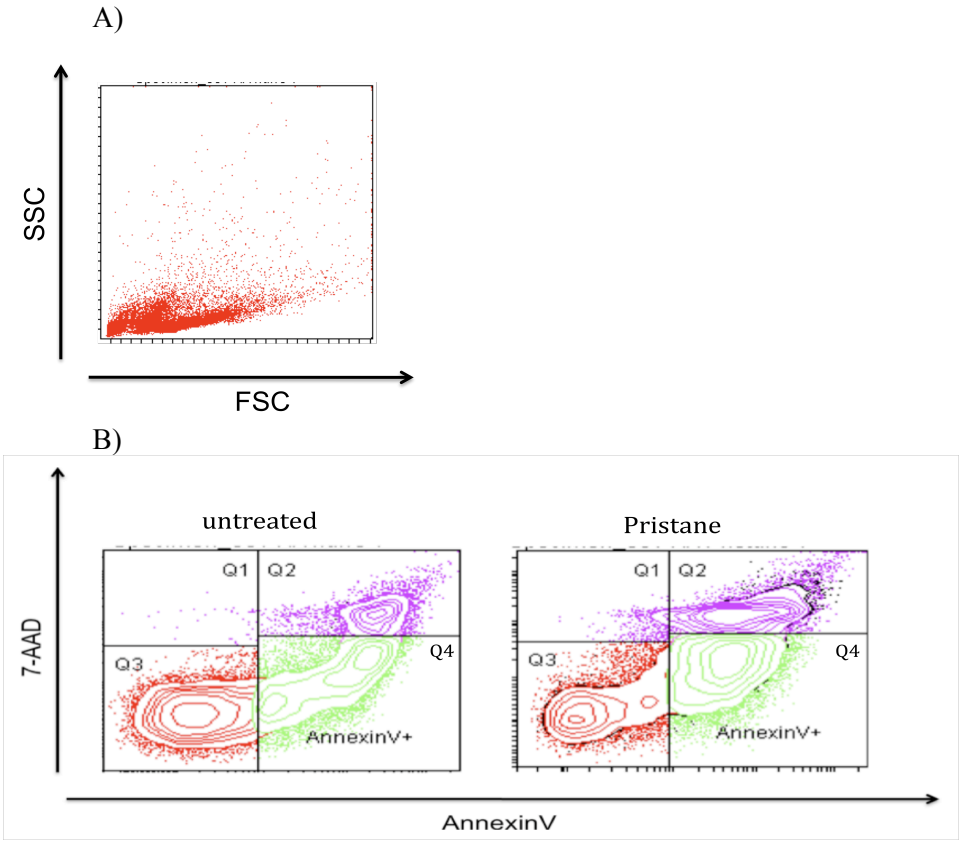

Figure 27 | Dot plots and contour plots from isolated and stimulated/unstimulated splenocytes stained with

7AAD and AnnexinV. Untreated and mineral oil treated cells were stained with 7AAD (corresponding to FL-3) and annexinV (corresponding to FL-1). Flow cytometry was performed with a FACSCalibur. No gate was set to analyze all cells, viable and dead (A). Gating was done for $7AAD^+$ cells and $annexinV^+$ cells. (B) Comparison between contour plots of untreated cells and pristane-treated cells. Q1 and Q2 refer to $7AAD^+$cells, Q3 to viable cells and Q4 to $annexinV^+$ cells.

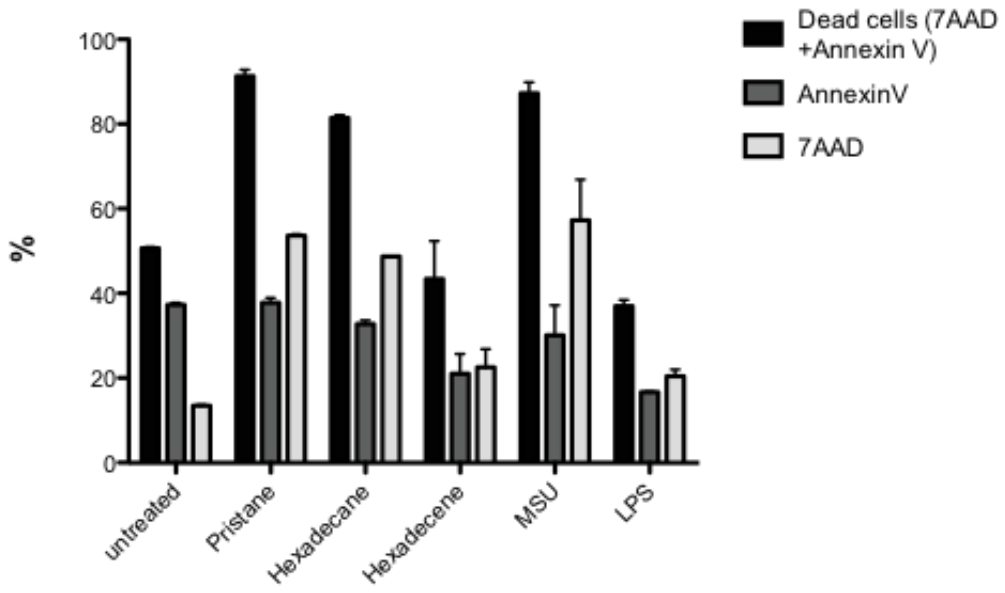

Figure 28 | Analysis of 7AAD and AnnexinV staining. Rat splenocytes were stimulated with oils complexed to β-cyclodextrin, MSU crystals, LPS, or left untreated. After o/n incubation staining with 7AAD and annexinV was performed and flow cytometry was done subsequently. The gated percentage of $7AAD^+$ cells, $annexinV^+$ cells and $7AAD^+$ /$annexinV^+$ cells are shown. Results are shown as mean ± SEM.

Inclusion complexes with pristane as well as MSU crystals induced cell death as seen from the higher percentages of annexinV/7AAD double positive cells as compared to untreated cells. Differences could also be observed between the different oils; hexadecane complexes induced slightly less cell death than pristane complexes while similar percentages of dead cells could be found in cultures treated with hexadecene complexes than in untreated cells. LPS stimulation of splenocytes led to slightly less dead cells in comparison to untreated cells (figure 28).

4.4.2. Intracellular IL-1β staining in combination with LIVE/DEAD cell stain *in vitro*

To answer the question whether there is a connection between cell death and IL-1β production (i.e. if the increased IL-1β levels in supernatants can be accounted for by IL-1β passively

released from dead cells), LIVE/DEAD cell stain was done in combination with intracellular IL-1β staining after stimulation *in vitro*.

Differentiated THP-1 cells were incubated o/n with oils complexed to β-cyclodextrin, staining was done with LIVE/DEAD cell stain (conjugated to FITC, corresponding to FL-1) and intracellular IL-1β antibody conjugated to PE (corresponding to FL-2) and analyzed by flow cytometry.

Dot plots of untreated cells and cells treated with inclusion complexes between β-cyclodextrin and pristane are shown in figure 29 and 30.

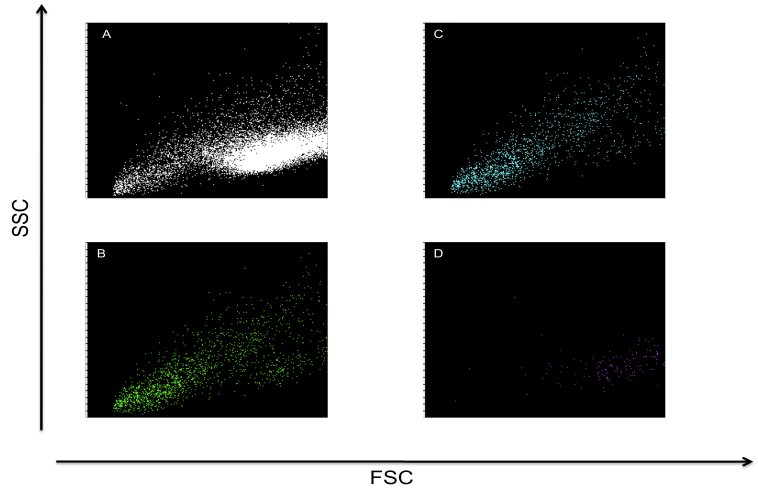

Figure 29 | Dot plots from untreated THP-1 cells stained for dead cells (LIVE/DEAD cell stain) and IL-1β. Untreated, differentiated THP-1 cells were stained with LIVE/DEAD cell stain (corresponding to Fl-1) and intracellularly for IL-1β conjugated to PE (corresponding to Fl-2). Flow cytometry was performed with FACSCalibur. Dot plots show all analyzed cells (A), gated IL-1β^{+} cells (B), gated LIVE/DEAD cell stain^{+} cells (C) and cells positive for IL-1β and negative for LIVE/DEAD cell stain (D).

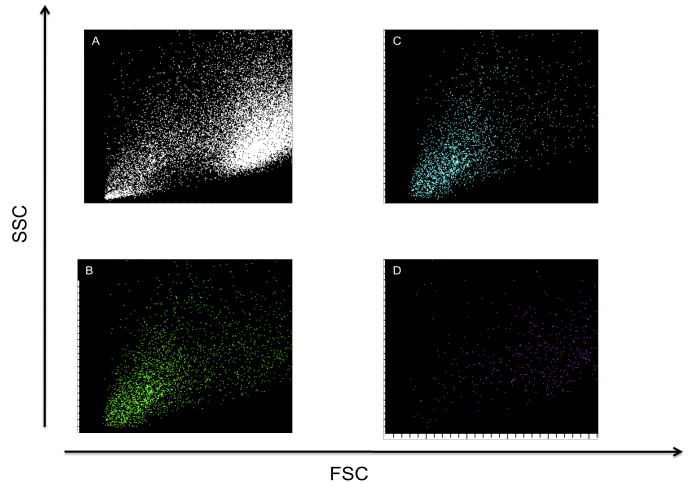

Figure 30 | Dot plots from THP-1 cells treated with pristane and stained for dead cells (LIVE/DEAD cell stain) and IL-1β. Pristane-treated, differentiated THP-1 cells were stained with LIVE/DEAD cell stain and intracellular for IL-1β conjugated to PE. Flow cytometry was performed with FACSCalibur Dot plots show all analyzed cells (A), gated IL-1β$^+$ cells (B), gated LIVE/DEAD cell stain$^+$ cells (C) and cells positive for IL-1β and negative for LIVE/DEAD cell stain (D).

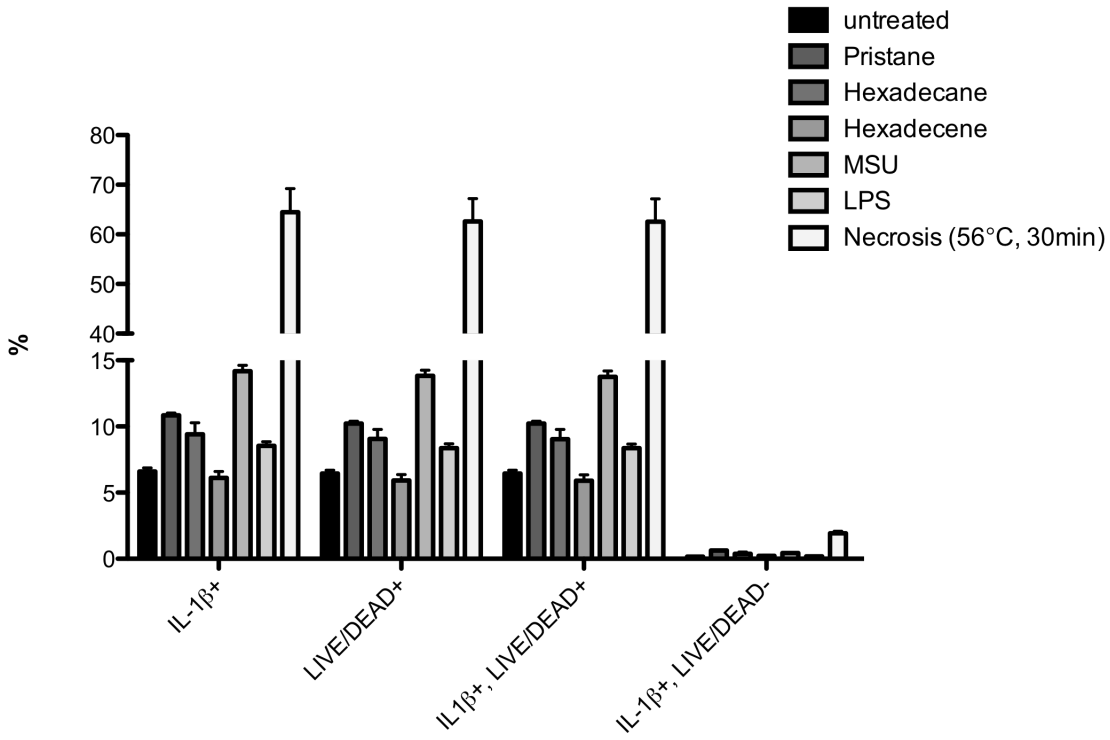

Figure 31 | Analysis of LIVE/DEAD cell stain in combination with intracellular IL-1β staining. The percentage of IL-1β$^+$ cells, LIVE/DEAD cell stain$^+$ cells, IL-1β$^+$ and LIVE/DEAD stain$^+$ cells and IL-1β$^+$ and LIVE/DEAD cell stain$^-$ cells in THP-1 cells treated as indicated. Results are shown as mean ± SEM.

As shown in figure 31, percentage of IL-1β+ cells was approximately twofold higher in cells treated with pristane complexed to β-cyclodextrin compared to untreated cells (see also dot

blots shown in figure 29B and 30B). A difference in IL-1β levels could also be observed between the different mineral oils. Pristane-treatment led to the highest percentages of IL-1β+ cells, followed by hexadecane. No elevation of percentages of IL-β+ cells compared to untreated cells was seen in cells incubated with hexadecene complexes. Percentages of IL-1β+ cells were also higher after treatment with MSU crystals and heat-induced necrosis, which served as a positive control for the staining. Similar results were obtained when gating was done for LIVE/DEAD cell stain positive cells, which led to the conclusion that almost all IL-1β positive cells were dead cells. Only few cells were viable and IL-1β positive (figure 29D and 30D). These findings were controversial regarding previous results because almost all IL-1β+ cells are dead and a staining artifact was considered.

4.5. Oxidative burst assay

The role of ROS in inflammasome activation is unclear. An oxidative burst assay was done to determine if mineral oils complexed to β-cyclodextrin induce ROS-production by themselves.

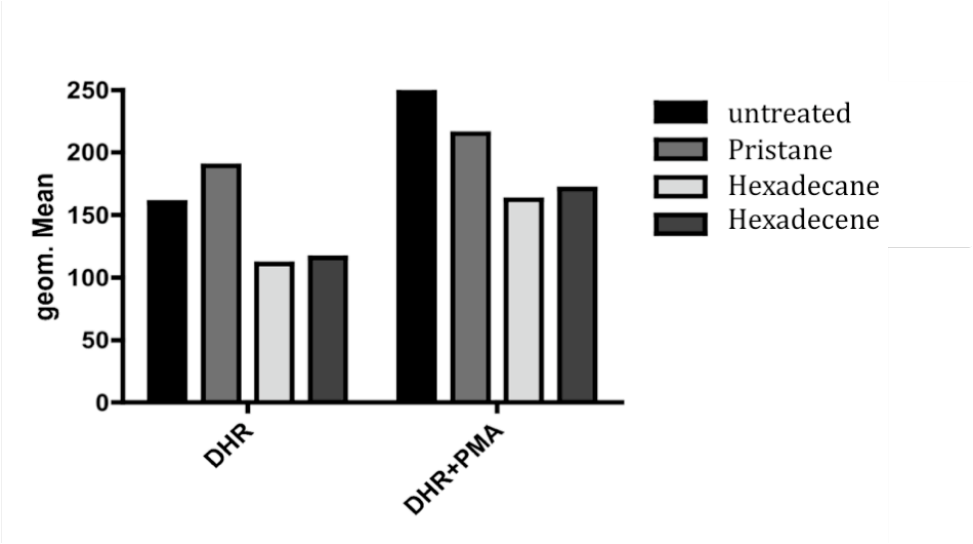

Figure 32 | Oxidative burst assay of THP-1 cells. Differentiated THP-1 cells were incubated o/n with inclusion complexes between β-cyclodextrin and pristane, hexadecane, or hexadecene. Oxidative burst assay was performed with dihydrorhodamine 123 (DHR) with and without PMA stimulation. Dihydrorhodamine oxidation to rhodamine induced by the respiratory burst of the cell was measured by flow cytometry on FL-1. N=1

Production of ROS was slightly higher after stimulation of the cells with pristane complexed to β-cyclodextrin while hexadecane and hexadecene-treated cells produced less ROS than untreated cells (figure 32). However, due to small sample number (N=1) interpretation of the obtained results is difficult.

4.6. Neutrophil extracellular traps

To investigate the response of neutrophils upon stimulation with mineral oils, formation of neutrophil extracellular traps (NETs) was determined. Neutrophils isolated from rat peripheral blood, either the DA.*Ncf1*^{DA} or the DA.*Ncf1*^{E3} congenic strain, were stimulated with β-cyclodextrin complexed to mineral oils (pristane or hexadecene) for 0h, 1h, 2h, and 4h. As positive controls PMA- and MSU-stimulated neutrophils were used. The DA.*Ncf1*^{DA} rat strain has a lower ability to produce ROS than the DA.*Ncf1*^{E3} strain.

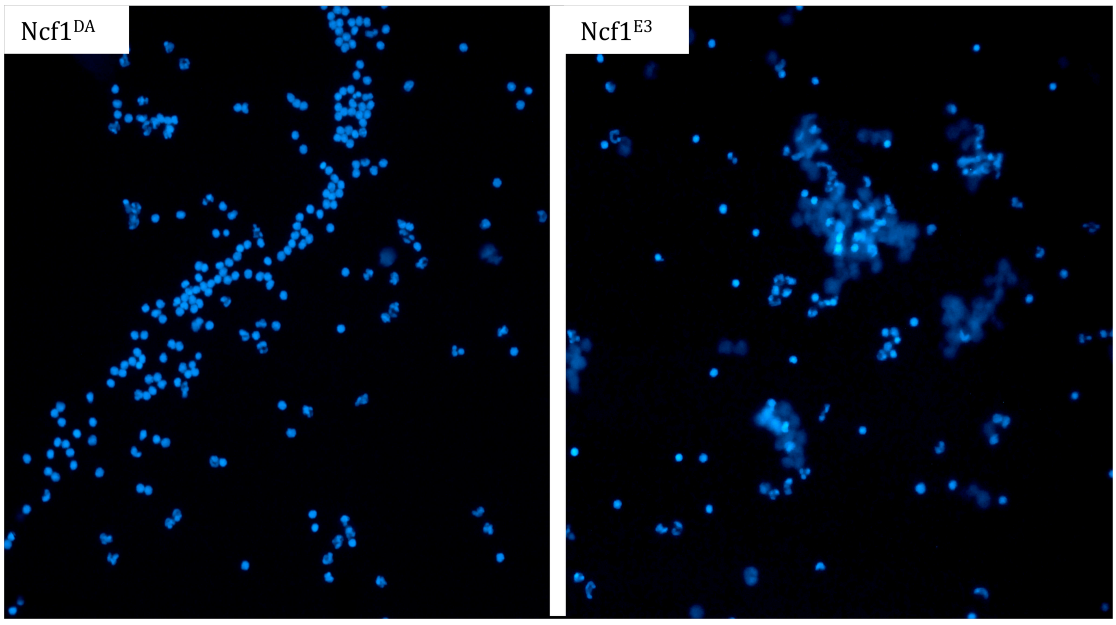

Figure 33 | NET formation in DA.*Ncf1* ^{DA} and DA.*Ncf1* ^{E3} rats. Isolated neutrophils from rat peripheral blood from either rat strain were stimulated for 2h with pristane complexed to β-cyclodextrin. Afterwards, cells were fixed with formaldehyde and cells were transferred to glass slides by cytospin and cells were stained with DAPI. Microscopic analysis was done on a fluorescence microscope. Picture shows neutrophils from DA.*Ncf1*^{DA} and DA.*Ncf1*^{E3} rats treated with pristane for 4 hours.

Microscopic NET analysis showed more NET formation in the oxidative burst-competent DA.*Ncf1*^{E3} strain upon treatment with pristane complexed to β-cyclodextrin (figure 33). These differences between the two tested strains were observed regardless of the stimuli. Furthermore, more NET formation was seen after treatment with pristane compared to hexadecene.

4.7. MSU crystals

MSU crystals were used as a positive control throughout many experiments included in this diploma thesis, often with lower outcome than expected. To investigate the reason for these insufficient results, purchased MSU crystals were compared to MSU crystals provided by the group of Martin Herrmann.

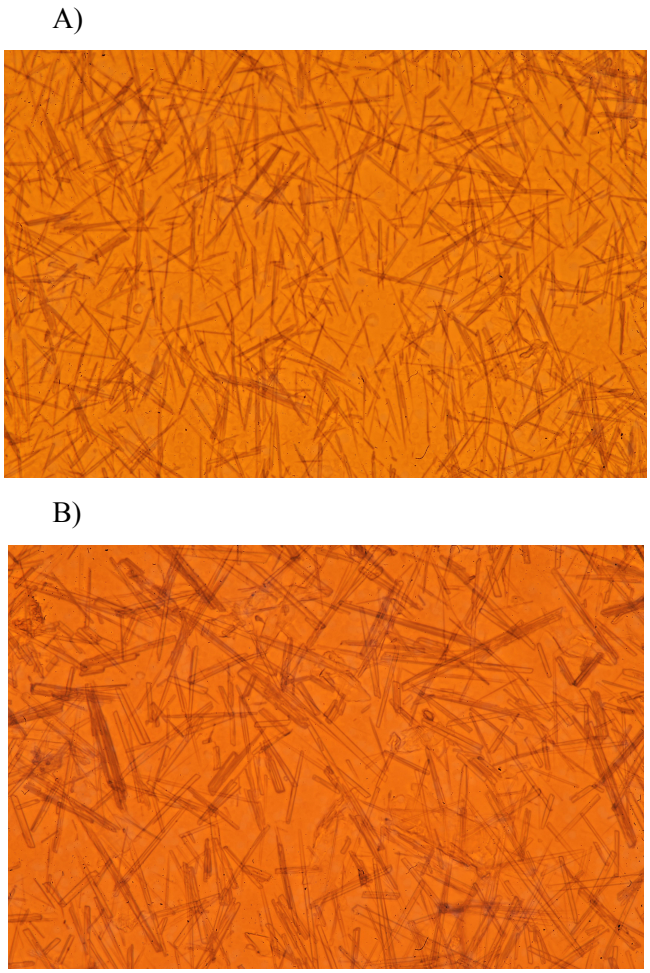

Figure 34 | Microscopic comparison between MSU crystals. A) Self-made crystals provided by the group of Martin Herrmann, University of Erlangen-Nuremberg. B) Purchased crystals from Sigma Aldrich.

Figure 34 shows big differences regarding size and shape between the crystals purchased from *Sigma Aldrich* (figure 34B) and self-made MSU crystals (figure 34A). Self-made MSU crystals were thinner and smaller compared to purchased crystals which could have an effect on the uptake of the crystals by the cells.

5. DISCUSSION

Inflammasome activation and the subsequent release of IL-1β are important in mediating inflammation. Dysregulated activation of these pathways is associated with various autoinflammatory diseases like gout or cryopyrin-associated syndromes. However, the role of the inflammasome in autoimmune diseases like RA and SLE is less clear.

PIA in DA rats is a well-established animal arthritis model and demonstrates a similar pathogenesis to human RA. In this model we could show that the serum cytokine levels of IL-1β mostly correlate with the disease course of arthritis in comparison to the other analyzed pro-inflammatory cytokines IL-6, TGF-β and TNF-α. Consistent with the known correlation of IL-1β levels with disease activity in human RA[75], serum levels of IL-1β in pristane-induced DA rats rose before disease onset and again increased previous to recurrence of the arthritis symptoms in the chronic phase (figure 13). However, even pristane-treated DA rats that did not develop arthritis symptoms (~5% of animals) had elevated serum IL-1β levels. Interestingly, in these phenotypically healthy pristane-treated DA rats, IL-1β levels also reflected the typical disease course of RA and even increased again at the time when the chronic phase was normally expected (unpublished data, Markus Hoffmann).

These findings indicate that IL-1β does not provoke the onset of arthritis in PIA but can be seen as a marker and may drive the outcome of the disease in combination with other cytokines.

At the molecular level however, the effect of pristane and other mineral oils is not yet clear. Nevertheless, a role for IL-1β in the pathogenesis of PIA seems to be likely and given by the fact that IL-1β is released upon inflammasome activation, the role of the inflammasome in PIA and subsequently in RA has to be determined. For this reason, the impact of different mineral oils at the molecular cell level was investigated *ex vivo* and *in vitro*.

MSU crystals, which are associated with gout, are known to activate the NALP3 inflammasome *in vitro* as well as *in vivo*.[26] With regards to these findings, MSU crystals (purchased from S*igma-Aldrich*) were used as a positive control in many of our experiments both *in vitro* and *ex vivo*. The effect of the crystal on different cell types (THP-1 as well as rat splenocytes) was surprisingly low (figure 16, figure 17) and investigations were made regarding the size and the shape of the crystals (figure 34). Comparison of purchased crystals from S*igma* was performed with self-made MSU crystals (kindly provided by the group of Martin Herrmann, University of Erlangen-Nuremberg) and crystals were found to differ in size and shape. This might be an explanation for the low IL-1β production upon stimulation

of the cells with MSU crystals. Cells might have been unable to uptake *Sigma-Aldrich* crystals, resulting in reduced inflammasome activation and subsequently unchanged IL-1β production. This is in line with previous results from Martin Herrmann's group and published data, demonstrating the necessity of the internalization of the crystals before IL-1β release.[76]

The role of IL-1β production was first investigated *ex vivo* using splenocytes from pristane-injected rats isolated on day 15 and day 21 after injection (figure 15). The observed higher IL-1β levels and the difference between day 15 and day 21 confirmed the results that IL-β increases *in vivo* during the acute phase of the disease. Stimulation of splenocytes from naïve DA rats with inclusion complexes between β-cyclodextrin and different mineral oils elevated IL-β levels significantly compared to untreated from naïve rats and PIA rats on day 15 and day 21 (figure 15B). After the finding that IL-1β levels are higher after treatment with β-cyclodextrin complexed to pristane, the impact of other mineral oils, hexadecane and hexadecene, on naïve rat splenocytes was tested (figure 16). A difference could be observed between the mineral oils, showing that hexadecane induces less IL-1ß secretion than pristane and that the non-arthritogenic oil hexadecene induces less IL-1β secretion than hexadecane. Thus these results suggest the most potent induction of IL-1β by the mineral oil pristane. These findings are consonant with the previous published data about arthritogenic adjuvants.[63]
Similar results were obtained upon treatment of the human monocytic cell line THP-1 with mineral oils complexed to β-cyclodextrin (figure 17). Consistent with the results from rat splenocytes, various oil stimulations by pristane, hexadecane and hexadecene of THP-1 cells caused differences in IL-1β production. Pristane as well as hexadecane elevated IL-1β levels significantly while hexadecene did not. In agreement with the fact that pristane and hexadecane are arthritogenic and hexadecene is not, these variable IL-1β levels in the cells are in line with the described arthritogenicity of the oils. Moreover, regarding the disease severity in DA rats, pristane induces higher arthritis scores in rats than hexadecane does.[63]
To further prove an inflammasome dependent mechanism, chemical inhibition was performed with two different inhibitors: z-VAD-fmk, a caspase inhibitor, and glibenclamide, a potassium channel inhibitor. Inhibition with z-VAD-fmk did not significantly reduce IL-1β levels in both experiments, *in vitro* and *ex vivo* (figure 16, figure 17), while glibenclamide showed an inhibitory effect on IL-1β production *in vitro* after incubation with pristane and hexadecane complexes. The inhibitory effect on *ex vivo* cells was achieved upon treatment with all three mineral oils. These findings were partially unexpected and the failure of z-VAD-fmk could not be explained.
Our previous results about the effect of mineral oils complexed to β-cyclodextrin on cytokine levels strengthen our hypothesis that pristane and other mineral oils have an effect on the

single-cell level. To mimic the *in vivo* situation better, oils were put directly on the cells without complexation to β-cyclodextrin. However, stimulation of naïve rat splenocytes with pure oils led to unexpected but interesting results: cells stimulated with oils after pre-incubation with the inhibitor z-VAD-fmk produced higher IL-1β levels than cells without inhibitors (figure 20). One possible explanation might be that splenocytes are too sensitive for this method and cells are dying before IL-1β production is induced. Furthermore, pre-incubation with the inhibitor z-VAD-fmk might prime the cells so that cells can produce IL-1β after stimulation with the oils. However, it is not known whether only mature IL-1β is detected by ELISA or both forms, the uncleaved pro-IL-1β and the cleaved IL-1β. Thus, the source of the detected IL-1β in supernatants could also be IL-1β passively released from dead or dying cells. Therefore it has to be considered that also necrotic cells in the supernatant lead to an increase of measured IL-1β levels.

In our *in vitro* system, findings were different: Stimulation of differentiated THP-1 cells directly with pure pristane led to a significantly elevated IL-1β secretion (figure 19). Regarding direct hexadecane and hexadecene stimulation, no significant difference in IL-1β levels were observed, which might be due to the fact that pristane is more arthritogenic than hexadecane and hexadecene is nonathritogenic or that pristane induces higher levels of cell death. Furthermore, also the lipid squalene that is arthritogenic as well induced higher IL-1β production. However, compared to the obtained levels upon incubation with pristane complexed to β-cyclodextrin, IL-1β levels were approximately 50% less (figure 17). These results suggest that complexation of mineral oils to β-cyclodextrin is the method of choice for delivering oils to mammalian cells although it might not reflect the *in vivo* situation best.

On the basis of our previous findings, the question was raised whether there is a connection between exposure to mineral oils and cell death. Indeed, increased apoptosis respectively necrosis seems to play a role in RA and other autoimmune diseases. Increased cell death is proposed to drive autoimmunity by releasing endogenous autoantigens such as nucleic acids, heat-shock proteins or citrullinated proteins. Thereupon, release of endogenous material, which is not cleared properly by macrophages, can lead to recognition of these molecules as autoantigens by the immune system. Cavali *et al.* have shown that pristane oil induces cell death by apoptosis in murine peritoneal cells *in vivo* as well as *in vitro* and in lymphoid cell lines. Thus, we tested whether mineral oils induce cell death in rat cells and the THP-1 cell line, subsequently possibly leading to autoimmunity and arthritis.[56]

It could be shown that cell death is more frequent after treatment of naïve rat splenocytes with pristane and hexadecane complexes. Again, a difference was detected between the arthritogenic oils. In contrast, hexadecene stimulation did not induce more cell death

compared to untreated cells (figure 28).

Similar findings were seen upon treatment of THP-1 cells with mineral oils and differences could also be seen among the oils: treatment with pristane led to more dead cells than hexadecane and no difference could be observed after hexadecene treatment compared to untreated cells (figure 31).

These data suggest that the arthritogenic oils pristane and hexadecane induce more cell death, which might reflect the situation in PIA.

As a result of the flow cytometry it was revealed that both cell types, THP-1 as well as rat splenocytes, showed an increase of their side scatter, reflecting increased granularity, after incubation with the complexes (figure 25, figure 26). Flow cytometry did not detect any more complexes after incubation with cells and so concluding from these findings it was assumed that cells might uptake complexes. Hence, phagocytosis of complexed oils by the cells was assumed to be the mechanism leading to an increase in granularity. However, it has to be considered that complexes could also stick to the cells and subsequently lead to the increased side scatter. Interestingly, differences regarding the side scatter of treated cells were observed among the oils. As the amount of complexes was adjusted before stimulation, cells were incubated with the same concentration regardless of the oil. However, analysis with flow cytometry showed that the complexes itself differ in size (figure 26) which indicates that the ingested complexes might have caused the increase of the side scatter but it could also be that the oil induced changes inside the cells, which led to a more granular morphology of the cell and can be detected via flow cytometry due to different refraction indexes of the oils. Again, pristane showed the highest impact followed by hexadecane. Little changes were observed after hexadecene treatment.

Apart from the elevated IL-1β production, it was supposed that pristane also activates inflammasome independent mechanisms. As shown in figure 21 and 22, mineral oils not only induce the release of cytokines dependent on caspase-1 activity but also cytokines such as TNF-α and IL-1α.

IL-1α production of naïve rat splenocytes as well as of THP-1 cells upon stimulation with complexed mineral oils correlated with the measured IL-β levels. It is known that IL-1α secretion requires the presence of IL-1β, which might cause the correlation between higher IL-1β levels and increasing IL-1α levels. Like IL-1β, IL-1α is produced as the inactive precursor pro-IL-1α. The pro-form remains in the cell but a portion of the unprocessed IL-1α is bound to the cell membrane.[77] Due to cell death, pro-IL-1α could be released into the supernatants and it is not clear whether the ELISA detected only mature IL-1α or pro-IL-1α as well (figure 21A and 22A).

Additionally, TNF-α levels were elevated in both splenocytes and THP-1 cells after treatment with mineral oils complexed to β-cyclodextrin. Interestingly however, hexadecene induced the highest TNF-α levels whereas pristane showed no significant increase compared to untreated cells (figure 21B).

Since a role of NETs in autoimmune diseases was implicated, NET formation was investigated in more detail. NETs are a neutrophil response to infections leading to the release of antimicrobial extracellular filaments made out of DNA, histones and other neutrophil proteins.[59] Differences in NET formation were seen between the oils but moreover, these differences were also observed between the rat strains DA and E3 (figure 33). Compared to DA rats, E3 rats have a higher ability to produce ROS. Given that E3 rats have milder form of arthritis after pristane injection it is assumed that ROS production has an anti-inflammatory effect [42]. Splenocytes, isolated from arthritis-susceptible DA rats formed less ore no NETs, whereas cells from the E3 strain showed NET formation upon treatment with mineral oils complexed to β-cyclodextrin. These findings suggest that NETosis, might be dependent on oxidative burst. However, pristane and other mineral oils did itself not provoke more oxidative burst *in vitro* (figure 32).

Taken together, our data demonstrate that stimulation of cells with mineral oil elevates IL-1β levels and that differences can be observed between different oils. It seems that pristane oil is the most potent inducer of IL-1β secretion compared to hexadecane and hexadecane, whereof the latter shows almost no induction of IL-1β. These findings are in line with the fact that pristane and hexadecane are known to be arthritogenic oils while hexadecene is not. However, it has to be considered that with the commercially available anti rat-IL-1β ELISA it is not possible to distinguish between secreted mature IL-1β and pro-IL-1β. The detected rise in IL-1β levels after application of oils might therefore be due to activation of the inflammasome, but also due to IL-1β passively released from dying cells. Cell death was shown to be more frequent upon stimulation with mineral oils and it suggests the important role of apoptosis and necrosis in systemic inflammation. However, it has to be investigated how IL-1β production and cell death are connected and whether there is an influence of IL-1β on cell death or vice versa. One might consider this as a 2-step mechanism: oils induce cell death quickly after stimulation, hence intracellular pro-IL-1β stores are released to the extracellular milieu. The second step induced by oils might involve the activation of the inflammasome and subsequent active release of mature IL-1β. Here, the method of choice would have been Western blot analysis to verify the results and to differentiate between pro-IL-1β and IL-1β. However, due to technical problems this issue could not be solved during this diploma thesis.

Moreover, it can be concluded that pristane as well as hexadecane and hexadecene not only activate the production of caspase-1 dependent cytokines but also other cytokines like IL-1α and TNF-α.

In conclusion, this work proposes that in PIA, pristane induces cell death and activates IL-1β production by means of inflammasome-dependent and -independent mechanisms that might drive the development of systemic inflammation in combination with other cytokines. The consequence of IL-1β activation is, among others, the expansion of naïve and memory T cells which is in accordance to the fact that pristane-induced arthritis is a T cell-dependent arthritis model. Still, the role of the inflammasome in this mechanism is not yet clear and more detailed analysis will be needed to verify the involvement of the inflammasome in the development of arthritis.

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7. ABSTRACT

The inflammasome is a cytosolic, multiprotein platform activated upon cellular infection or stress. It is a component of the innate immune system, expressed in myeloid cells and activates an inflammatory cascade, triggering inflammatory caspases and subsequently leading to maturation of proinflammatory cytokines such as interleukin (IL)-1β, IL-18, or IL-33. Dysregulation of these pathways is associated with various autoinflammatory diseases like gout or cryopyrin-associated syndromes that are characterized by episodes of recurrent fever. However, the role of the inflammasome in autoimmune diseases like rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) is so far not clear.

Animal models have been useful for defining possible mechanisms in the pathogenesis of human autoimmune diseases. Pristane and other mineral oils like incomplete Freund's adjuvant (IFA) and hexadecane are well known adjuvants to induce autoimmune phenotypes, mostly arthritis and lupus-like disease after injection in experimental animals. Although these animal models are often used to study autoimmune diseases *in vivo*, their way of action is incompletely understood.

It was therefore the aim of this thesis to study the impact of the arthritogenic mineral oils pristane and hexadecane as well as the non-autoimmunogenic control oil hexadecene *ex vivo* on isolated rat splenocytes as well as *in vitro* using the human monocytic cell line THP-1. To circumvent the problem of the poor immiscibility of the oils in aqueous cell culture medium, molecular encapsulation into β-cyclodextrin was done. Alternatively, oils were applied to cells directly. Supernatants were analyzed for the cytokines IL-1β, IL-1α, and TNF-α. Moreover, cells were analyzed by flow cytometry to study morphological changes as well as cell death induced by mineral oils.

Stimulation of cells with mineral oil elevated IL-1β levels, with pristane oil inducing a higher increase in IL-1β secretion than hexadecane. In contrast, hexadecene caused almost no induction of IL-1β. Pristane as well as hexadecane and hexadecene also triggered the production of the caspase-1 independent cytokines IL-1α and TNF-α.

Morphologically, cells showed increased side scatter indicative of a more granular phenotype upon stimulation with pristane and hexadecane. Furthermore, cell death was accelerated in cells treated with pristane compared to hexadecane or hexadecene.

In conclusion, autoimmunogenic mineral oils such as pristane or hexadecane trigger inflammasome-dependent and -independent IL-1β production and induce cell death, with

pristane that is more effective for disease induction *in vivo* also having bigger effects *in vitro*. In combination with other cytokines, these mechanisms might contribute to the development and maintenance of systemic inflammation in autoimmune diseases.

8. ZUSAMENFASSUNG

Das Inflammasom ist ein zytosolischer Proteinkomplex, welcher als Teil des angeborenen Immunsystems in myeoliden Zellen exprimiert wird. Nach Stimulierung durch Infektionen oder intrazellulären Stress kommt es zur Aktivierung einer inflammatorischen Kaskade. Im Zuge dessen werden inflammatorische Caspasen angeregt welche die inaktive Vorstufen von den proinflammatorischen Zytokine Interleukin (IL)-β, IL-18, oder IL-33 spalten und diese damit aktiviert.

Eine Dysregulation dieses Signalweges ist assoziiert mit auto-inflammatorischen Krankheiten wie etwa Gicht oder dem Cryopyrin-assoziierte periodisches Syndrom welches durch wiederkehrende Fieberschübe gekennzeichnet ist. Ungeklärt ist jedoch bis heute die Rolle des Inflammasoms in Autoimmunerkrankungen wie der rheumatoiden Arthritis (RA) oder dem systemischen Lupus erythematosus (SLE).

Tiermodelle sind hilfreich um Krankheitsentstehung und -verlauf von Autoimmunerkrankungen *in vivo* zu untersuchen. Durch einmalige Injektionen von Pristan oder anderen Mineralölen wie etwa inkomplettem Freund'schem Adjuvans (IFA) oder Hexadecan, werden Arthritis und Lupus-ähnliche Erkrankungen im Tiermodell hervorgerufen. Trotz der häufigen Verwendung dieser Modelle in der Praxis, um autoimmune Erkrankungen *in vivo* zu untersuchen konnte der zu Grunde liegende molekulare Mechanismus noch nicht eindeutig erklärt werden.

Diese Diplomarbeit hatte deshalb das Ziel, die Auswirkungen von arthritogenen Mineralölen wie Pristan und Hexadecan und dem nicht arthritogenen Öl Hexadecen *ex vivo* an Milzzellen, isoliert von Ratten, und *in vitro* an der humanen monozytischen Zelllinie THP-1 zu testen.

Aufgrund der schlechten Löslichkeit von Ölen im wässrigem Medium wurden die Öle mit dem Zucker β-Cyclodextrin komplexiert. Zusätzlich wurde versucht die Öle direkt zu applizieren. Die Überstände der behandelten Zellen wurden hinsichtlich der Zytokine IL-1β, IL-1α und TNF-α analysiert. Morphologische Untersuchungen mit Hilfe von Durchflusszytometrie wurden durchgeführt, und weiters die Auswirkung der Öle auf den Zelltod getestet.

Die Stimulation der Zellen mit Mineralölen führte im allgemeinen zu einer erhöhten IL-1β Produktion und Sekretion. Pristan induzierte, im Vergleich zu Hexadecan, einen höheren Anstieg von IL-1β. Im Gegensatz dazu hatte eine Stimulierung mit Hexadecen keine Wirkung auf die IL-1β Sekretion. Weiters konnte gezeigt werden, dass sowohl Pristan als auch

Hexadecan einen Anstieg der Caspase-1 unabhängigen Zytokine IL-1α und TNF-α induzieren.

Morphologisch betrachtet wiesen Zellen nach Behandlung mit den Ölen Pristan und Hexadecan eine Zunahme des side scatter auf, welches mit einer höheren Granularität der Zelle korreliert. Im Zuge dieser Arbeit wurde auch ein Zusammenhang zwischen Zelltod und Pristan hergestellt.

Zusammenfassend konnte gezeigt werden, dass die Mineralöle Pristan und Hexadecan sowohl Inflammasom-abhängige als auch Inflammasom-unabhängige Produktion von IL-1β induzieren. Gleichzeitig führt die Applikation von Pristan zu erhöhtem Zelltod, wobei der stärkere Effekt von Pristan *in vivo* im Vergleich zu anderen Mineralölen auch *in vitro* beobachtet werden konnte. In Verbindung mit anderen Zytokinen könnten diese Mechanismen zur Entstehung und Aufrechterhaltung von Autoimmunerkrankungen beitragen.

9. APPENDIX

9.1. List of abbreviations

´	minutes
´´	seconds
°C	degrees Celsius
AIA	antigen induced arthritis
AIM2	absent in melanoma 2
AP-1	activator protein 1
APC	antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a CARD
ATP	adenosine triphosphate
CARD	caspase recruitment domain
CGD	chronic granulomatous disease
CIA	collagen induced arthritis
CINCA	chronic infantile cutaneous neurological articular syndrome
CPPD	calcium pyrophosphate dihydrate
D-MEM	Dulbecco's modified eagle medium
DA	dark agouti
DAMP	danger associated molecular pattern
DAPI	4', 6-diamidino-2-phenylindole
ddH_2O	double distilled water
DHR-123	dihydrorhodamine-123
DMSO	dimethyl sulfoxide
DPI	diphenyleneiodonium chloride
DTT	1,4 Dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence activating cell sorting
FCAS	familial cold autoinflammatory syndrome
FCS	fetal calf serum
FIIND	function to find domain
g	gram

h	hours
HBSS	Hank’s balanced salt solution
HIN	interferon inducible
IL	interleukin
IL-1R	IL-1β receptor
IS	immune system
kDa	kilodalton
l	liter
LPS	lipopolysaccharide
LRR	leucine rich repeats
M	molar
mA	milliampere
MAPK	mitogen-activated protein kinase
ml	milliliter
mM	millimolar
MSU	monosodium urate
MWS	Muckle-Wells syndrom
NACHT	NAIP, CIITA, HET-E and TP-1-domain
NALP	NACHT, LRR and PYD-containing protein
NETs	neutrophil extracellular traps
NF-κB	nuclear factor 'kappa-light-chain-enhancer' of activated B-cells
NLR	NOD-like receptor
nm	nanometer
nM	nanomolar
NOD	nucleotide-binding/oligomerization domain
NOMID	neonatal-onset multisystem inflammatory disease
o/n	overnight
OIA	oil induced arthritis
PAMP	pathogen-associated molecular pattern
PBS	phosphate buffered saline
pen/strep	penicillin/streptomycin
PIA	pristane-induced arthritis
PMA	Phorbol-12-myristate-13-acetate
PRR	pattern recognition receptors
PVDF	polyvinylidenfluorid
PYD	pyrin domain
PYHIN	pyrin and HIN200-containing proteins

RA	rheumatoid arthritis
RIG	retinoic acid inducible gene I
ROS	reactive oxygen species
rpm	revolutions per minutes
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLE	systemic lupus erythematosus
TBS	tris buffered saline
TLR	toll-like receptor
TNF	tumor necrosis factor
V	volt
µg	microgram
µl	microliter
µM	micromolar

9.2. List of figures

9.3. List of tables

10. CURRICULUM VITAE

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